

A calm view of video violence

Studies of violence in the media and its effects on people are clouded by overheated rhetoric and exaggerated claims. More clarity is needed, both in the science and in the way it is discussed.

There are good reasons to be troubled by the violence that pervades the media. Movies, television and video games are full of gunplay and bloodshed, and one might reasonably ask what's wrong with a society that presents videos of domestic violence as entertainment. Of course, the same questions could have been raised about watching *Macbeth*, or *Punch and Judy*. Let's face it, people have always enjoyed watching other people's mayhem.

Most researchers agree that the causes of real-world violence are complex. A 1993 study by the US National Academy of Sciences listed "biological, individual, family, peer, school, and community factors" as all playing their parts. And a 2001 report by the US surgeon general concluded that "the preponderance of evidence indicates that violent behavior seldom results from a single cause; rather, multiple factors converging over time contribute to such behavior".

Viewing abnormally large amounts of violent television and video games may well contribute to violent behaviour in certain individuals. The trouble comes when researchers downplay uncertainties in their studies or overstate the case for causality. Sceptics were dismayed several years ago when a group of societies including the American Medical Association, the American Psychological Association and the American Academy of Pediatrics tried to end the debate by issuing a joint statement: "At this time, well over 1000 studies ... point overwhelmingly to a causal connection between media violence and aggressive behavior in some children."

Freedom-of-speech advocates accused the societies of pandering

to politicians, and even disputed the number of studies (most were review articles and essays, they said). When Jonathan Freedman, a social psychologist at the University of Toronto in Canada, reviewed the literature, he found only 200 or so studies of television-watching and aggression. And when he weeded out "the most doubtful measures of aggression", only 28% supported a connection.

The critical point here is causality. The alarmists say they have proved that violent media cause aggression. But the assumptions behind their observations need to be examined. When labelling games as violent or non-violent, should *Pac-Man* gobbling a ghost really be counted as a violent event? And when experimenters measure physiological arousal, or record the time it takes game players to read 'aggressive' or 'non-aggressive' words from a list, can we be sure what they are actually measuring? The intent of the new Harvard Center on Media and Child Health, to collect and standardize studies of media violence in order to compare their methodologies, assumptions and conclusions, is an important step in the right direction.

Another appropriate step would be to tone down the crusading rhetoric until we know more. Several researchers write, speak and testify prolifically on the threat posed by violence in the media. That is, of course, their privilege. But when doing so, they often come out with statements that the matter has now been settled, drawing criticism from colleagues. In response, the alarmists accuse critics and news reporters of being duped by the entertainment industry. Such clashes help neither science nor society. ■

The grand challenges facing physics

It would be easy to delay the prioritization of major new projects at US physics laboratories, but it needs to be done.

It is more than a decade since the US Department of Energy (DOE) last set out a programme of major new facilities for its network of laboratories. The intention of Ray Orbach, head of the DOE Office of Science, to draw up such a list should be welcomed by researchers.

The DOE is understandably tight-lipped about its plan (see page 357). It has little enough money to support current operations, and there is a very real risk that publishing the list will trigger conflict between the winners and losers. But a list of priorities will soon be needed. With most individual investigators based at universities, the main function of the department's laboratory network is to build and house facilities that the universities themselves could not afford. Such projects take upwards of a decade to plan and construct.

The choices to be made leave many researchers understandably nervous. In the context of frozen budgets — a context that the Office of Science has largely endured since the cancellation of the Superconducting Super Collider in 1993 — facilities must be built at the short-term expense of the very people who will use them.

But with no major facility planned since the Spallation Neutron Source, now under construction at the Oak Ridge National Laboratory in Tennessee, a backlog of potential opportunities is accruing. Orbach is right to bite the bullet and prepare a new plan. It is understandable that he wants to be cautious in discussing its contents,

in advance of their approval by his boss, energy secretary Spencer Abraham, and by the bean-counters at the White House Office of Management and Budget.

In their different ways, Europe and Japan are also finding it difficult to build large facilities, faced with problems in garnering the necessary support. The fusion project ITER and a linear particle collider (as well as upgrades at CERN's Large Hadron Collider) will be global projects, but there is no consistent framework for these to be considered by the nations that might want to build them. The Organisation for Economic Cooperation and Development's Global Science Forum remains the most credible body for such discussions, but its influence will need to grow if it is to fulfil this role.

In the meantime, Orbach can draw some encouragement from Congress's recent support for his office (see page 361). Progress seems to have been made in convincing law-makers that the DOE's physics programmes, in particular, are a valuable part of the nation's scientific portfolio. Extra impetus should also come from the gradual expansion of the National Institutes of Health's role in equipping large facilities, such as synchrotron light sources, that are also used by biologists. After a difficult spell, this process is the best opportunity that the DOE has had for years to plan properly for its laboratories' futures. ■





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Physics poised for sizeable gains in energy department's wish-list

Geoff Brumfiel, Washington

The US Department of Energy (DOE) is developing an ambitious plan to build a new generation of large scientific facilities during the next two decades.

Over the past six months, the head of the DOE's Office of Science, Ray Orbach, has been collecting a list of proposed experiments and facilities that would range in cost from tens of millions to billions of dollars. The list now includes some 50 projects, mostly in the physical sciences, and supporters say it could provide direction for the science office for years to come.

But before the list can be prioritized and released, it must win the support of the energy department's highest officials and pass muster with the president's budget office, which is attempting to hold down government spending in the face of record budget deficits.

Researchers at the laboratories involved are eager to learn how their projects are faring in the prioritization process, but Orbach has declined repeated requests to discuss its progress. The facilities plan is currently undergoing a stringent review within the upper levels of the department, according to those close to the process.

The DOE is the largest supporter of



Digging deep: (from left) Ray Orbach, Senator Lamar Alexander, Spencer Abraham and William Madia, director of Oak Ridge National Laboratory, break ground for the lab's nanotechnology facility.

physics in the United States, and Orbach's office spends more than \$3 billion a year. Most of that goes on a network of laboratories that house many of the nation's largest

scientific facilities, such as the Tevatron accelerator at Fermilab near Chicago, which discovered the top quark in 1995. The department last outlined a comprehensive facilities

US observation initiative calls for fresh view of Earth

Tony Reichhardt, Washington

The United States is convening an international summit in Washington DC next week to tout plans to consolidate scientific observations of the Earth — and to help repair its reputation as a foot-dragger in global climate change.

Attendees at the meeting on 31 July will be asked to sign a declaration calling for increased sharing of data from instruments on the ground, in the air and in space. The summit will be followed by meetings of a working group to begin developing a ten-year plan for an integrated Earth-observation system, which would help to standardize data

from sensors operated by different nations, as well as identify gaps in those data.

Participants will include US secretary of state Colin Powell and half-a-dozen other US cabinet members, along with ministers from most leading industrial nations and representatives of the World Bank and the World Meteorological Organization.

Ray Williamson, an expert on Earth-observation policy at George Washington University in Washington DC, says that some contentious issues could surface at the meeting — such as who would set the requirements for an integrated system. Europeans already have programmes with

similar goals, he says. They may also be wary of any US effort to dominate the field.

The United States also faces scepticism from those who suspect that its enthusiasm for collecting more data on climate change is simply "a way of putting off making some hard decisions" on greenhouse gases, Williamson says.

But Anthony Janetos, director of the global-change programme at the Heinz Center, a think-tank in Washington, says that given the difficulties of coordinating large observation programmes, "the reaffirmation of a US commitment to an integrated system is enormously important".

plan 20 years ago, in an exercise that led to the construction of a string of projects. Although it still starts up some medium-sized facilities — such as the nanotechnology facility for which Orbach and his boss, energy secretary Spencer Abraham, broke ground last week at Oak Ridge National Laboratory in Tennessee — it has no overall plan for larger ones.

Last December, Orbach sent out a letter to advisory groups asking them to suggest new facilities that the directorate could build. “Creating these facilities for the benefit of science is at the core of our mission,” the letter read.

The response was enthusiastic, according to University of Oregon chemist Geraldine Richmond, who chairs the department’s basic energy sciences advisory committee. Among the facilities recommended by Richmond’s committee is a series of new light sources to probe materials and molecules.

At the top of the nuclear physicists’ list is the Rare Isotope Accelerator, which would carry out experiments on highly unstable nuclei in an effort to reproduce nuclear reactions inside stars and supernova explosions. Planning for the machine has been hampered by budget problems and uncertainty over when it will be built, says Richard Casten, a nuclear physicist at Yale University who heads the nuclear science advisory group. “My feeling is that maybe what Orbach’s doing can change that,” he says.

At a cost of about \$800 million, the isotope accelerator would be one of the more ambitious projects under consideration, but it is by no means the most expensive. Also being considered are a \$5-billion experimental fusion reactor known as ITER, and a \$6-billion linear collider for high-energy physics.

Martha Krebs, who served in Orbach’s position under the Clinton administration from 1993 to 1999 and is now a consultant in Los Angeles, says: “Putting out a preferred list will be very difficult to do.” She tried to draw up a similar list of facilities during her tenure, but ran into difficulties with the energy secretary and the White House Office of Management and Budget, whom she says wouldn’t approve a plan that called for billions of dollars in additional spending.

At that time, support in Congress was weak for both the energy department and its science office — but that may be changing, Krebs says. The House of Representatives has just approved a 6.5% increase for DOE science (see page 561), and congressional staff members who oversee the department are eager to see what facilities plan the department can come up with. “I’m dying to see how they’re going to handle this,” says one. ■



Green issue: scientists say that transgenic crops’ effects on farmland are their most pressing concern.

UK experts map out route to licensing transgenic crops

Jim Giles, London

There are no human-health grounds for holding up the planting of transgenic crops in Britain, an expert panel has told the UK government.

But the panel said that the crops could have adverse environmental impacts, and should only be grown after case-by-case assessments of the risks. Its findings, released on 21 July, are expected to influence the government’s decision on whether to license some transgenic crops for commercial planting later this year.

The panel was chaired by the government’s chief scientific adviser, David King. Its work is part of a three-pronged assessment of transgenic crops by the UK government, which has also included a report issued on 11 July on the crops’ economic impact, and a public consultation involving 500 meetings held around the country.

The process is being watched closely because British consumers’ rejection of the technology is influencing its adoption in many other countries. “This will be influential internationally,” says plant scientist Mark Tester of the University of Cambridge.

But it remains unclear whether the panel’s tentative green light for the technology will be enough to open the door to British cultivation of transgenic crops any time soon. Prime Minister Tony Blair has advocated the technology, but his unpopularity in the aftermath of the Iraq war may reduce the likelihood that he can persuade consumers, or environmental protesters, to accept it.

The panel, which included representatives from the biotechnology industry and conservation organizations as well as university scientists, struggled to reach a consensus,

its members say. On the way, they digested more than 600 papers, lost a panel member, and survived a series of last-minute revisions that threatened to scupper the entire project.

“It’s a minor miracle that the report got put together,” says panel member Mike Gasson, head of food-safety science at the Institute of Food Research in Norwich. Carlo Leifert, an expert in organic agriculture at the University of Newcastle-upon-Tyne, quit the panel last month.

The panel’s report makes some concessions to critics of transgenic food, including, for example, the observation that allergens produced by transgenes may not be spotted during regulatory screening, and may only emerge once a crop is widely grown. But another fear often raised by environmental groups — that transgenic crops could give rise to herbicide-resistant ‘superweeds’ — was played down in the report.

Members were also split on whether extensive growth and consumption of such crops in the United States constitute evidence that they are safe to eat. The panel eventually agreed that the available research shows risks to human health to be “very low”.

A large-scale study of the impact of herbicide-tolerant transgenic crops on biodiversity, conducted by a team of UK-based scientists (see *Nature* **412**, 760–763; 2001), is currently undergoing peer review, and the panel said it was reluctant to draw conclusions about environmental risks in the meantime. The panel said, however, that “the most important issue is [the crops’] potential effect on farmland and wildlife”, and pledged to update its report after the biodiversity study is published. ■

♦ www.gmsciencedebate.org.uk

Dispute over data privacy halts cancer study

David Cyranoski, Tokyo

The brakes have been slammed on a major cancer-research project, after the Japan Medical Association (JMA) accused its leaders of failing to provide adequate protection for participants' privacy.

The incident illuminates the distrust between physicians and clinical researchers in Japan which, observers say, continues to dog efforts to bolster the country's clinical research capacity.

The cancer project seeks to study the respective roles of lifestyle and genetics in determining susceptibility to major cancer types. Its planners want to collect blood samples, as well as information on diet, exercise and sleeping habits, from 100,000 subjects.

The project began in May with a survey of 6,000 citizens in Kumano-cho, a small town near Hiroshima. Project organizers planned to start taking blood samples in August.

But on 16 July, the Tokyo-based JMA, reacting to enquiries from Kumano-cho residents, sent a letter of complaint to the education ministry, which funds the project. The letter said that the project's data-collection process could not ensure that the personal information gathered would remain private.

"They were using ordinary citizens — people who had no legal responsibility to protect the information — to go to people's



houses and collect it," says Rintaro Sawa, a member of the JMA's board of trustees. Sawa claims that the study took advantage of the fact that people in Hiroshima are used to answering such questions for radiation-fallout studies.

The project's leaders have now told the education ministry that they will postpone the study for a year while they try to find a new way of collecting the data. One of the project leaders, Kei Nakachi, a cancer epidemiologist at the Radiation Effects Research Foundation in Hiroshima, says he accepts the JMA's critique. "We want to be honest

and open and look at our problems," he says.

But some researchers are perplexed by the watchdog role that the JMA seems to be assuming over their work. The JMA says it has no choice, as a law enacted in May last year to ensure the security of personal information fails to require researchers to monitor their own procedures (see *Nature* **417**, 689; 2002). "There's no one else to do it," says Sawa. From August, the JMA will establish a committee to oversee the use of personal information and medical samples in research.

The JMA is also monitoring a project to create a database of genetic information using 300,000 samples from patients who suffer from various diseases or drug side-effects (see *Nature* **423**, 209; 2003).

Yusuke Nakamura, a genomicist at the University of Tokyo and that project's leader, says he plans to spend ¥8 billion (US\$70 million) of the project's ¥20-billion five-year budget on proper methods for collecting and storing data, including the establishment of computer firewalls to protect the data, and training of medical professionals to collect samples.

But Nakamura worries that the JMA's aggressive stance on privacy will make participants uneasy, and scare them away from his study. "We have been very careful. I don't want to lose the patients' trust," he says. ■

African project seeks model solution to water shortage

Quirin Schiermeier, Munich

Water supply is one of the toughest issues facing the world's poorest countries. But an innovative project in West Africa could offer a way forward, according to hydrologists involved in the scheme.

German and Ghanaian researchers are working together to create a model of how meteorological, environmental, social and economic variables affect the Volta basin. By 2006, they hope to be able to use the model to make practical decisions about water management in the region.

The Volta basin comprises parts of Ghana, Burkina Faso and several adjacent countries. Agriculture is the main source of income in this area, and population growth is now putting pressure on land and water resources.

The scheme is part of the GLOWA (Global Change in the Hydrological Cycle) initiative, set up by the German government in 2001 to improve water management in several different river catchments. Other pilot studies are taking place on the Elbe and Danube rivers in Germany, the Jordan River Basin, the Wadi Drâa in

Morocco and the River Ouémé in Benin.

In the first phase of the Volta project, researchers assessed the water flows and patterns across the Volta basin. In the second phase, they plan to use this information to build a model that will enable local planners to assess the impact of their actions on water management, agriculture, ecology and power supply. The German government this month approved €10 million (US\$11.3 million) to support this second stage.

"This is the most comprehensive science-led approach ever tried in West Africa to guarantee the effective allocation of water resources," says Daniel Adom, a water engineer and executive secretary of the Ghanaian Water Resources Commission (WRC).

"We envisage a smart scientific toolbox for modelling, at different scales, the likely impact of different water-management options on power production, industry, agriculture and human health," says GLOWA Volta's scientific coordinator, Nick van de Giesen, a hydrologist at the Center for Development Research at

the University of Bonn in Germany.

Looming water crises were the subject of the 3rd World Water Forum in Japan (see *Nature* **422**, 251–256; 2003). But experts think that the real action will take the form of relatively small initiatives, targeted at specific river basins.

Ghana, with its relatively strong scientific infrastructure and stable political system, is an ideal location for the project, van de Giesen says. The country has decades of experience with major water projects, and the dam on the river at Lake Volta supplies most of its electricity. But the growing population and a shortening rainfall season mean that Ghanaians are drawing off more of the river's flow for household use and for farm irrigation, which is relatively new in the region.

This pressure has led to growing political conflict over the use of water, leading the WRC to revise its water-management and regulation practices in the Volta basin. But, says van de Giesen, "the knowledge base is narrow — and that's where GLOWA Volta comes in". ■

♦ www.glowa-volta.de

Brain region linked to post-terror stress

David Cyranoski, Tokyo

Researchers studying the long-term effects of the 1995 sarin gas attack in the Tokyo underground have unearthed some intriguing clues about what controls different people's susceptibility to the psychological after-effects of shock.

Hidegori Yamasue, a psychiatrist at the University of Tokyo, has been looking at images of the brains of survivors of the attack. He found that of 25 survivors studied, the volume of an area of the brain known as the anterior cingulate cortex (ACC) was smaller, on average, in the nine who were diagnosed with post-traumatic stress disorder. The degree of size reduction correlated with the severity of the symptoms.

The finding, reported this week in the *Proceedings of the National Academy of Sciences* (100, 9039–9043; 2003), could help to clarify a difficult area of neuroscience. Several previous studies searching for a link between the size of a different area of the brain, the hippocampal region, and stress symptoms have produced inconclusive results.

But Yamasue has found signs of a much stronger link between the size of the ACC and the occurrence of post-traumatic stress. Many of those hurt in the Tokyo attack have shown symptoms of the disorder, ranging from nightmares and flashbacks to fear of riding trains.

Yamasue used an innovative magnetic resonance imaging technique that divides the brain into about a million small elements for computer analysis, enabling him to look at the whole brain at once. The results showed no correlation between the stress



Many survivors of the 1995 sarin gas attack in Tokyo later suffered from post-traumatic stress.

symptoms and the size of the hippocampal region. "We looked over the whole brain and only the ACC region was different," he says.

A recent study of medical workers who served with the US army in Vietnam also suggested a correlation between the size of the ACC and post-traumatic stress (S. L. Rauch *et al. NeuroReport* 14, 913–916; 2003). But Roger Pitman, a Harvard psychiatrist and co-author of the study, believes that the size of the hippocampal region could correlate with post-traumatic stress in victims of "chronic, long-standing" trauma, including the subjects of most of the previous studies.

But researchers still don't know whether a smaller brain region is a result of the stress, or a pre-existing condition, perhaps genetic in

origin, that made the subject more likely to display symptoms. Investigating possible links between hippocampal size and post-traumatic stress, Pitman has studied twins of whom one had experienced trauma (M. W. Gilbertson *et al. Nature Neurosci.* 5, 1242–1247; 2002). His results tentatively suggest that the size factor may be a pre-existing condition but "the jury is still out on this," he says.

Linking post-traumatic stress with specific areas of the brain could eventually aid the treatment of its victims. "If we understand the changes in brain structure and function we can develop better treatments targeted at specific brain pathways," says Gerardo Villarreal, a psychiatrist at the University of New Mexico. ■

Museum trustees quit after row over sale of artefacts

Rex Dalton, San Diego

The board of trustees at a leading museum in the western United States has resigned, following an outcry over the sale of some of its collection to help cover operating costs.

The Museum of Northern Arizona (MNA) in Flagstaff, best known for its archaeology and palaeontology collections, will hold a meeting of its supporters on 26 July to elect new trustees to try to end what local scientists describe as a year of financial and administrative turmoil at the museum.

On 11 July, the museum's trustees and its director, Robert Baughman, said they would resign with effect from 26 July, after leading donors and museum supporters set up a petition to force them out.

Baughman and the trustees declined to comment on the resignations, but a museum statement said that they were intended "to

restore confidence in the institution".

Over 75 years, the museum has curated one of the nation's leading collections of Native American artefacts and art, together with dinosaur and geological specimens.

But along with many other US museums, the MNA has been going through tough financial times (see *Nature* 423, 575; 2003). Early last year, when it was running out of operating funds, the museum's leadership made the decision to declare some pieces of the art and archaeology collection 'non-performing assets' and sell them for about \$850,000 to a private dealer.

Among the items sold were half a dozen sandpainting textiles by Hostenie Klah, an esteemed ceremonial practitioner in the Navajo tribe who died in 1937. "This never should have happened," says Susan Brown McGreevy, an authority on Klah's work

and former director of the Wheelwright Museum of the American Indian in Santa Fe, New Mexico. "It was an act of total irresponsibility."

When details of the sale became known earlier this year, a museum donor, Cynthia Perin, filed a complaint with the American Association of Museums (AAM). Perin says that the AAM has opened an investigation, as its ethics code forbids the sale of part of the collection to generate operating revenue. Perin says she is "appalled" by the museum's actions, including the scaling-back of its geology department.

AAM-accredited museums, such as the MNA, should not consider their collections as assets, says Ed Able, the AAM's president. Able declined to confirm that an investigation was under way, but says that he has never known a museum board to resign en masse. ■

HIV drug resistance triggers strategic switch

Erika Check, Washington

High rates of drug resistance are causing infectious-disease experts to recommend that doctors change the way they use medications against HIV.

A study released on 16 July by Charles Boucher, a virologist at Utrecht University in the Netherlands, at a meeting in Paris of the International AIDS Society (IAS), found that 10% of Europeans newly infected with HIV carry a strain that is resistant to medication.

The study confirms warnings that HIV drug resistance is on the rise. Last August, for example, a team led by researchers from the University of San Diego found that the number of patients newly infected with HIV in North America who carried resistant virus had jumped from 3.4% in 1995–98 to 12.4% in 1999 and 2000 (S. J. Little *et al. N. Engl. J. Med.* **347**, 385–395; 2002).

The growth of resistance has profound implications for AIDS treatment globally, researchers say. On 14 July, the US Department of Health and Human Services released a set of updated guidelines for anti-retroviral treatments, cautiously recommending for the first time that doctors test for drug resistance in newly infected patients at the outset of treatment, to make sure that the drugs they prescribe will work.

A panel of experts convened by the IAS on 1 July published similar recommendations (*M. S. Hirsch et al. Clin. Infect. Dis.* **37**, 113–128; 2003). The IAS panel said that all



Nigerian AIDS patients benefit from drugs donated by Americans who have become resistant.

patients who have been infected for less than two years before beginning treatment should be tested for drug resistance. Virologists have found that drug-resistant forms of HIV can survive in patients for at least two years, and are still studying whether resistance can last longer than that.

Most doctors use drug-resistance tests only in HIV patients who have had several rounds of drug treatment and stopped responding. This is because the tests cost up

to \$800 per patient, and are sometimes difficult for clinicians to interpret.

But better tools are becoming available to interpret the tests, and as drug resistance becomes more common, testing will become more cost-effective. One analysis has found that testing costs no more than dosing patients with drugs to prevent infections when resistant strains of HIV are common (*M. C. Weinstein et al. Ann. Internal Med.* **134**, 440–450; 2001).

“If the prevalence of resistance is 10% or 12%, and you know that resistance can persist for some time, the case for early testing is much stronger,” says Dan Kuritzkes, director of AIDS Research at Brigham and Women’s Hospital in Boston, Massachusetts, and a member of the IAS panel.

The growing rate of resistance raises worries about the pace of drug discovery. This year, three new AIDS drugs were approved by the US Food and Drug Administration. So far, experts say, they have kept up with drug resistance. But the future is hard to predict.

“What we don’t know is whether resistance is still growing or whether it’s essentially plateauing,” says Anthony Fauci, director of the US National Institute of Allergy and Infectious Diseases.

Some health officials are calling for more detailed guidelines on which drugs to use in different patients — especially in poor countries, where treatment is not yet widespread. Observers say it will be difficult to get doctors in these countries to submit to such rules.

“If we’re going to implement these rules in Africa, we should also do it in Europe,” says IAS president Joep Lange. But even in rich countries, he says, doctors are used to prescribing drugs they are familiar with. “They don’t think there might be alternatives,” he says.

US budgets for physical endeavour

Geoff Brumfiel, Washington

Physical sciences in the United States could be set for their largest funding boost for years, as Congress adds money to the 2004 budget numbers proposed by President George W. Bush back in February.

Appropriations subcommittees in the House of Representatives have voted to give two major research agencies — the National Science Foundation (NSF) and the Office of Science at the Department of Energy (DOE) — increases of about 6.5% next year, taking their budgets to \$5.6 billion and \$3.5 billion, respectively. On 18 July, the full House endorsed the DOE increase.

Bush had proposed a rise of 3.2% for the NSF, which funds most non-biomedical university grants in the United States, and 1.4% for the DOE’s science office, which supports most physics research.

The increases are good news for fields that have lately been eclipsed by a doubling of funding at the National Institutes of Health, the main life-science agency. “I think

there is a recognition that the future of the country depends on the physical sciences,” says Michael Lubell, director of public affairs at the American Physical Society.

It remains to be seen whether the proposed increases will be agreed by the Senate this summer, or be in the final budget bills that both bodies are due to agree by 1 October. “We love the increases,” says Senator Pete Domenici (Republican, New Mexico), who chairs the Senate subcommittee responsible for the DOE. But he notes that the House bill gave money to research by cutting water management projects, which have considerable support on his committee.

And not every major agency supporting the physical sciences is faring so well in the current budget round. The House appropriators have offered NASA an increase of only 1.1%, to \$15.5 billion. Meanwhile the House has approved a 1% increase, and the Senate a 4.7% cut, in basic and applied research at the Department of Defense.

Improved SARS test cuts final case count in United States by half

Washington Severe acute respiratory syndrome (SARS) infected only about half as many Americans as was originally thought. The US Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, has revised its official count of 418 down to 211 cases.

Originally, diagnosis was based on clinical criteria including the sudden onset of respiratory distress and high fever. These symptoms overlap with those of other illnesses such as influenza, but SARS can now be differentiated using tests that detect antibodies against the SARS virus.

The World Health Organization says that other countries should similarly use these tests to get a more accurate picture of how many victims the outbreak claimed, and to establish a more accurate profile of the disease's symptoms. But it warns that diagnosis based on clinical symptoms should not be made too restrictive, or positive cases may slip through the net.

♦ www.cdc.gov/ncidod/sars

Women's career award crosses gender divide

Washington He walks like a man and talks like a man — indeed, Philip Stahl is a man. So why has he won the American Society for Cell Biology's annual Women in Cell Biology Senior Career Recognition Award?

Because, the society says, Stahl has done an outstanding job of promoting women scientists' careers — from campaigning for a daycare centre at his medical school to mentoring female cell biologists who have gone on to prominent positions. It is the first time since the award's inception in 1986 that a man has been honoured.

Stahl, who is chair of cell biology and physiology at Washington University School of Medicine in St Louis, studies signal transduction. He credits his career in science to a female professor at West Liberty State College in West Virginia who convinced him, as an undergraduate, to change from accounting to biology. Stahl believes that good mentoring is crucial to encouraging more women to stay in science. "It's a matter of planning, encouragement and support," he says.

US visa problems curb rise in foreign students

Washington Record numbers of foreign graduate students are being blocked from studying at US universities because of immigration problems, according to a new study from the American Institute of Physics.

Amateur anglers cast as nature monitors

London Britain's anglers, hikers and other nature-lovers are being turned into scientific experts in an effort to monitor the status of declining plants and animals. The scheme, called 'Amateurs: the Experts', is being run by the Natural History Museum in London and the government conservation body English Nature.

One project is enlisting anglers to survey populations of river insects such as mayflies (pictured). The John Spedan Lewis Trust for the Advancement of the Natural Sciences in Stockbridge, Hampshire, has run identification workshops for river-keepers and anglers since 1997. Now, with input from museum specialists, the scheme has been expanded, with data being supplied for national recording schemes.

A survey of Britain's ancient elm trees — dubbed Elm Map — has been also been tied to the UK Ramblers' Association's annual Welcome to Walking Week, which begins on 20 September. Fifty of the 350 organized walks will involve data logging for Elm Map.



GEOFF DORE/NATUREPL.COM

During the 1990s, the number of foreign students enrolled in US graduate physics programmes rose steadily. By 2001, it had actually exceeded the number of American students. But since the terrorist attacks of 11 September 2001, the percentage of international students entering the programmes has dropped by almost 10%, according to the survey of 187 US science departments.

Roughly 20% of all international students accepted to study physics in the United States last year were unable to begin their studies on the intended date because of immigration difficulties, the survey found.

♦ www.aip.org/statistics

NASA to set up safety centre in wake of shuttle crash

Washington With a report due in August that is expected to criticize its handling of safety concerns before the accident that destroyed the space shuttle Columbia, NASA last week announced the creation of an independent engineering and safety centre at its Langley Research Center in Virginia.

The new centre will have the authority to stop launches if it perceives a problem, and will operate independently — which is one reason that it was not located in Texas or Florida, where the shuttle is managed and prepared for launch. Some 250 personnel throughout NASA will be affiliated with the centre, although only a few will be located at Langley.

Still undecided is how the new centre will coordinate its activities with NASA's existing safety offices, which involve 887 civilian government workers and more than 3,100 contractors and military employees.

Tattoo effects may be more than skin deep

Munich As fashionable Europeans bask in the sun, displaying their tattoos and piercings, a less fashionable working group of dermatologists and toxicologists is considering whether body art can injure their health.

Wolfgang Bäumler, a dermatologist at the University of Regensburg, Germany, has studied dyes collected from tattooing studios. He found that some contained allergens or carcinogenic agents that could be activated during laser tattoo removal.

The European Commission, which has convened the working group, wants to regulate the licensing, labelling and use of materials as well as hygiene at tattooing studios and staff training. Bäumler and others are now undertaking further studies to learn more about long-term health effects of tattooing and body piercing.



Don't change your mind: laser removal could activate harmful agents in tattoo dyes.

ROMEO RANOCO/REUTERS

Synthetic sex cells

Some pioneering biologists are trying to grow eggs and sperm in the lab. In doing so, they're entering a technical and ethical minefield. Carina Dennis reports.

Boy meets girl, sperm meets egg. It's a timeless tale, but one that is increasingly being played out in the culture dish — witness the million-plus children who have so far been conceived by *in vitro* fertilization (IVF), and the widespread application of assisted reproduction to livestock breeding.

IVF may just be the start. In the future, sperm and eggs may not just be brought together in a culture dish — they might also be grown there. Many of the biologists working towards this goal want to understand the developmental processes that give rise to mammalian sex cells. Some also hope that the technology will boost the clinical prospects of 'therapeutic cloning' — which will require a supply of human eggs to turn a patient's own cells into tissues to replace those lost to age, injury or disease. Others see applications for lab-grown sex cells in animal breeding.

For the time being, few researchers are enthusiastic about using lab-grown sperm and eggs for human reproduction — although this prospect is already overshadowing public debate about the work. Just a few weeks ago, many media commentators were outraged at reports from a fertility conference in Spain on attempts to culture human eggs from fetal ovaries. "Your real mother was aborted baby," ran a lurid headline in one British tabloid newspaper.

Even if the ethical questions can be successfully addressed, growing sperm and eggs in the lab poses formidable technical challenges. The production of sex cells is a complex, multi-stage process (see figure,

opposite). One major hurdle is coaxing the cells to halve their number of chromosomes, which involves a form of cell division known as meiosis. Another complication is that, under natural circumstances, sex cells do not grow and mature on their own: sperm development is nurtured by helpers known as Sertoli cells; the precursors of eggs are cushioned and nourished by granulosa cells.

Most attention is focusing on eggs, rather than sperm, as these are the scarcer resource. A prize bull, for instance, produces millions of sperm in a single ejaculate, but a prime milking cow will naturally produce only a handful of calves in her lifetime. Yet a female mammal's ovaries contain thousands of cells that have the potential to develop into eggs. Were it possible to culture these cells in the lab, elite cows could yield many more embryos, which would then be implanted into surrogate mothers.

Early developments

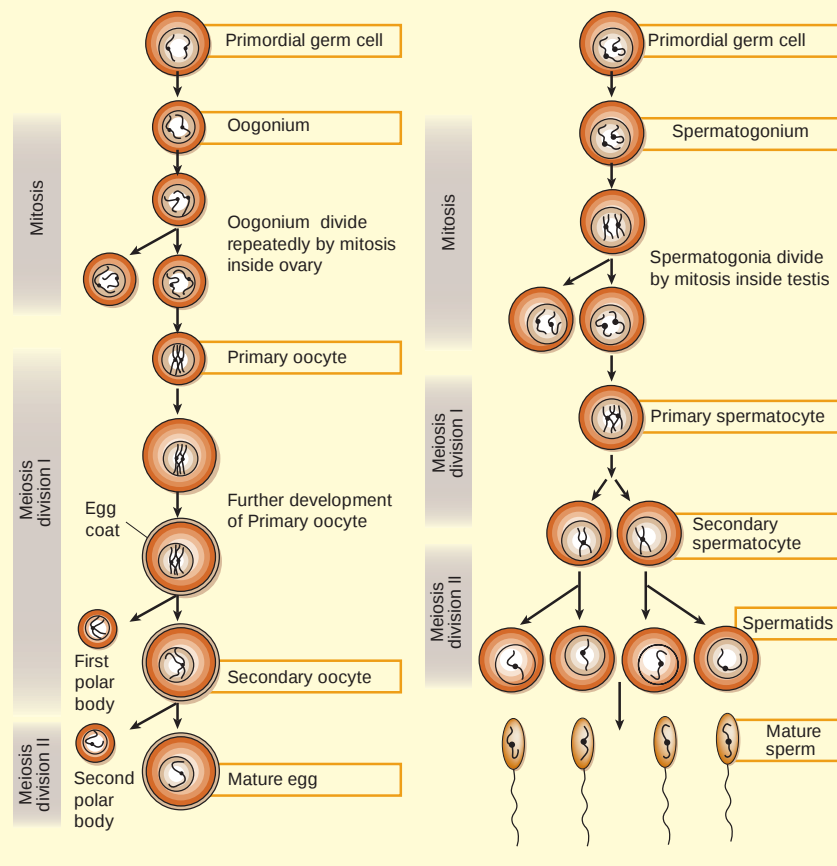
John Eppig, a developmental biologist at the Jackson Laboratory in Bar Harbor, Maine, has been trying to grow mammalian eggs in the lab for many years. He reported his first success back in 1996 (ref. 1). Eppig extracted ovaries from newborn mice and cultured them for eight days, before dissecting out precursors of eggs, known as oocytes, and their associated granulosa cells. He then grew these complexes for a further 14 days in a variety of culture media, obtaining the best results with one containing epidermal growth factor — which promotes the growth of granulosa cells and their interaction with oocytes. Many of

the resulting eggs began to develop after being fertilized, but the 190 two-celled embryos transferred into female mice generated only one live pup. Nicknamed Eggbert, this mouse was sickly, suffering from obesity and neurological problems in adulthood.

Since then, Eppig has honed his techniques by modifying the nutrient and hormonal supplements that he adds to the cultures. In a paper published online in December last year², he reported the birth of 59 live mice from 1,160 two-celled embryos — about one-third of the success rate achieved with IVF embryos generated from normal mouse eggs. The mice are now adults, and so far "look healthy and normal", says Eppig.

A few months earlier, a team led by Izuho Hatada of Gunma University in Japan achieved similar results by culturing oocytes from the ovaries of mouse fetuses about halfway through their gestation³. Hatada's team produced 16 live pups, but their lab-grown cells would only undergo the final stages of meiosis if they were first fused with naturally grown mature eggs stripped of their own genetic material. To ease problems with the supply of mammalian eggs, therefore, Hatada's technique needs further work.

Eppig and Hatada's experiments have so far attracted little public comment. But the potential for controversy, should similar techniques be applied to human reproduction, was underlined by the hostile reaction to related work reported on 30 June at a meeting of the European Society for Human Reproduction and Embryology in Madrid. Researchers led by Tal Biron-Shental of Meir



Path to life: the natural steps in egg and sperm creation; experts hope to adapt this process in the lab.

Hospital in Kfar Saba, Israel, had taken slices of ovarian tissue from aborted human fetuses, grown them in culture for a month, and found that the follicles containing immature oocytes had continued to develop. Donated human eggs are in short supply for IVF. But Biron-Shental's suggestion that aborted fetuses might be used to meet this demand was greeted with widespread revulsion — and in some countries, such as Britain, the practice would in any case be banned.

Even if ethical objections to using aborted fetal material could be removed, lab-grown eggs may not be safe for human reproduction. "Culturing methods have improved, but we are a long way from matching Mother Nature," says Eppig. Lab-grown eggs are smaller than normal and seem to be less capable of being fertilized and of developing as embryos.

One concern is that culturing could result in deleterious genetic changes. In unpublished work, Patricia Hunt of Case Western Reserve University in Cleveland, Ohio, has already seen changes in chromosome numbers in cultured mouse oocytes. "The final stages of oocyte growth are critical," she says. "I suspect it isn't the length of culture time, as much as the culture conditions."

There are also concerns that culturing could disrupt the subtle mechanisms that normally ensure that some genes are activated in an embryo depending on whether they are inherited from the mother or father. These 'epigenetic' marks are laid down in developing sex cells, and their disruption can give rise to developmental abnormalities.

While some biologists refine their techniques for culturing eggs, other researchers are working on the male side of the equation. A team led by John Parks at Cornell University in Ithaca, New York, has taken the precursors of sperm cells from the testes of newborn bulls, and grown them in culture together with Sertoli cells. After several weeks, many cells had developed into spermatids, the round cells from which mature, mobile sperm later develop⁴. More recently, Ryuzo Yanagimachi and his colleagues at the University of Hawaii in Honolulu went one step further. They grew mouse spermatids by a similar method, injected them into mouse eggs, and found that they produced normal, fertile offspring⁵.

Cultural mission

Researchers led by Martin Dym, a reproductive biologist at Georgetown University School of Medicine in Washington DC, have managed to generate spermatids in culture without the aid of Sertoli cells⁶ — although to do this, they had to engineer the precursor cells to contain a gene for an enzyme called telomerase, which allows cells to divide indefinitely in culture. Dym is now collaborating with Yanagimachi to see if his spermatids can yield live pups. "We have embryos, but no implantation yet," says Dym.

So far, no one has managed to get spermatids to develop into mature sperm in a simple lab culture. "The big hurdle is the final step," says Toshiaki Noce of the Mitsubishi Kagaku Institute of Life Sciences in Tokyo, who is also working on techniques to grow



Fat chance: Eggbert, the first mouse born from a lab-cultured egg, suffered obesity later in life.

sperm in the lab. It is possible, however, to coax spermatids to mature into fully fledged sperm by transplanting them into animals' testes, using techniques pioneered in the mid-1990s by Ralph Brinster of the University of Pennsylvania in Philadelphia⁷⁻⁹. They might even be matured in testis tissue grafted under an animal's skin¹⁰.

Most researchers, when trying to grow mammalian sperm or eggs in the lab, start with precursor cells extracted from ovaries or testes. But in May, a team led by Hans Schöler of the University of Pennsylvania's New Bolton Center in Kennett Square set the field abuzz by reporting the production of egg-like cells from mouse embryonic stem (ES) cells¹¹. Schöler and his colleagues allowed the stem cells to grow at high densities until some proliferated into floating clumps, which were then transferred into new cultures.

The researchers found that some of these aggregations developed into structures similar to ovarian follicles — a large, egg-like cell surrounded by smaller ones, reminiscent of granulosa cells. Adding hormones called gonadotropins caused the 'eggs' to be expelled from the follicle, just as in natural ovulation. What's more, the egg-like cells seemed to have entered meiosis — although it remains to be shown whether their chromosomal number was halved. Schöler has also yet to show that his artificial eggs can be fertilized and give rise to mouse pups.

Other researchers are trying to grow sperm from ES cells. In Tokyo, Noce has nudged cultures of mouse ES cells down the developmental pathway that leads to sperm production. This work has yet to be published, but Noce claims that his cultures yielded male primordial germ cells, the earliest precursors of sperm. After being matured into sperm in the testes of adult mice, the cells seemed to be able to fertilize eggs. But the proof will be in the birth of healthy pups. "Even abnormal sperm can activate eggs," warns Noce, "so I don't know whether the sperm are normal or not at this stage."

George Daley, a stem-cell biologist at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts,

Fresh eggs: structures that closely resemble ovarian follicles have been created from stem cells.

claims to have derived spermatocytes, a later stage in sperm production, from mouse ES cells. Again, the work is unpublished, and Daley warns that it is difficult to confirm his achievement from the cells' molecular characteristics. But he adds: "We have experiments under way to see whether we can isolate sperm that will actually fertilize eggs."

Lab-grown sex cells could readily find applications in animal breeding. In addition to boosting the number of offspring that could be bred from prize female animals, such cells could provide a new means of creating transgenic animals. Mice carrying targeted genetic modifications can be created by altering ES cells, introducing these into pre-implantation embryos and then breeding from the resulting mice to obtain animals with the desired genetic characteristics. For other species, it may be possible to achieve similar results by altering the precursors of sperm, maturing them in the lab, and then using them for IVF. "This would be especially useful for those animals that don't have good ES-cell lines, such as rats and pigs," says Dym.

The prospect of creating a prolific supply of lab-grown eggs would also provide a boost for experiments in therapeutic cloning, which will otherwise be severely limited by the supply of donated human eggs. Here, the idea would be to 'reprogramme' a patient's cells by fusing them with lab-grown eggs that have been stripped of their own chromosomes. After culturing the resulting embryos

for a few days, it should be possible to derive human ES cells, perfectly matched to the patient, that could potentially be coaxed into creating a variety of cells and tissues.

For many researchers trying to grow sperm and eggs, the main goal is to unlock the natural secrets of mammalian sex-cell development. Scientists would like to know the cocktail of growth factors and hormones that turn stem cells into precursor sex cells. "This is important because many of the mutations that affect adult fertility have been found to affect genes that act early in germline development," says Peter Donovan, a stem-cell biologist at Thomas Jefferson University in Philadelphia. "It could tell us an awful lot about the parameters that affect human and domestic-animal fertility."

Body doubles

Rather than mimicking the natural processes of sex-cell formation, however, some researchers are trying to trick normal body cells into behaving like sperm or eggs. For instance, Orly Lacham-Kaplan, a reproductive biologist at the Monash Institute of Reproduction and Development in Melbourne, Australia, has injected adult male cells into mouse oocytes that still contained two sets of chromosomes. The oocytes spat out half of the chromosomes from each of their constituent nuclei, and began to develop like fertilized eggs¹². Whether the resulting embryos are viable remains to be shown,

however. "We are in the process of testing whether offspring will be born and whether they will be normal," says Lacham-Kaplan.

Meanwhile, Gianpiero Palermo, an IVF expert at Cornell University's Weill Medical College in New York City, has injected adult cell nuclei into eggs stripped of their chromosomes, and discovered that these nuclei can subsequently be made to expel half of their chromosomes^{13,14}. For both mouse and human cells, the resulting 'eggs' can be fertilized — although, again, Palermo has yet to produce any offspring using his method.

Palermo's technique requires eggs, so this will not solve problems with the supply of eggs for either animal or human reproduction. Instead, his eventual goal is to provide fertility treatments for older women whose ovaries are no longer able to produce viable eggs. "One-third of our IVF patients are over 40 years of age," says Palermo. "More than half of their eggs can be abnormal."

Such talk of using artificially produced eggs for human reproduction alarms many researchers in the field — especially as those produced by Palermo's method may be even more prone to abnormalities than those grown by processes that mimic the natural process of sex-cell formation. "It is simply ripping the genetic material asunder with no evidence that normal meiosis has taken place," argues Eppig.

Other researchers point out that the cells that give rise to sperm and eggs possess mechanisms to minimize the accumulation of mutations. Most of the body's cells, however, lack these mechanisms, and so hardly represent ideal genetic stock from which to breed a baby. "It seems risky," says Donovan.

Questions are beginning to be asked about the safety even of existing techniques for assisted reproduction^{15,16}. Given our current state of knowledge, most experts believe that any attempt to use lab-derived eggs or sperm for human reproduction — however they are produced — would be an act of extreme folly.

Carina Dennis is Nature's Australasian correspondent.

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Playing with fire?

The latest computer games involve pretty gruesome scenes — severed limbs and drive-by shootings are standard fare. But opinion is divided on whether such games spark real-life violence. Tony Reichhardt investigates.

How about this for two contrasting views of the same phenomenon? When Craig Anderson watches teenagers playing violent video games such as *Doom* and *Grand Theft Auto*, he believes that seeds of aggression are being sown in their minds. Anderson, a psychologist at Iowa State University in Ames, says that experiments he and others have carried out show that players become more likely to harbour aggressive thoughts and show aggressive behaviour. He sometimes opens articles about media violence with ominous reminders of the 1999 Columbine High massacre in Littleton, Colorado. The Columbine killers, it seems, were big fans of *Doom*.

Kevin Durkin, a psychologist at the University of Western Australia in Crawley, observes teenagers playing arcade games, even violent ones, and sees something entirely different: kids having a good time. Whatever aggression the players show towards each other is generally good-natured, and accompanied by laughter.

“The main type of aggression was robust treatment of the equipment,” he noted dryly in a 1999 study for the Australian government¹. Computer games, he concluded last year², can be “a positive feature of a healthy adolescence”.

Contrasting views are not unusual in media-violence research. In one corner are parents’ groups and politicians, who worry that the games’ violence fuels real-life aggression, and say that the majority of research confirms such fears. Lined up against them are the researchers who side with Durkin, together with freedom-of-speech advocates. The evidence is weak, they counter, and their opponents use dubious statistics to make their point.

So who is right? The answer is far from clear, partly because the two sides are engaged in a war of words that can be as combative as some of the games being studied. Researchers who deny a link between violence and computer games have, for example, had their work challenged on the grounds that they received funding from the entertainment

industry. Others point out that some supporters of a link make money by advising parent-focused media watchdogs. The answer may be out there, but locating it amid the controversy is difficult. *Mortal Kombat* indeed.

Study stand-off

The public split between the two sides centres on their interpretation of existing studies. Although the vast majority of work on violence in the media has focused on television, Anderson and supporters say that we already know enough about computer games to be concerned. Together with former Iowa State colleague Brad Bushman, now at the University of Michigan in Ann Arbor, Anderson analysed 35 video-game studies carried out as of 2000. The research, the pair argue, shows that video-game violence “is correlated with aggression in the real world”³.

Typical of the studies is one that Anderson carried out with Karen Dill, a psychologist at Lenoir-Rhyne College in Hickory,

North Carolina. College students played violent and non-violent games, and then competed against each other in another game in which they tried to push a button faster than their opponent. If they won, they got to blast the loser with a loud noise. Those who played the violent game blasted their opponents for about a tenth of a second longer than non-violent gamers, but only when they had been blasted themselves in the previous round. "Playing a violent video game increased the aggressiveness of participants after they had been provoked by their opponent's noise blast," the authors concluded⁴.

But Durkin's review of the literature led him to downplay the threat from video games: "Despite much debate about the consequences of playing games with aggressive content, the evidence available to date to support claims of harmful effects upon children is modest." Ditto Lillian Bensley and Juliet VanEenwyk, epidemiologists at the Washington State Department of Health in Olympia, who published a review of computer-game literature in 2000. "The research evidence is not supportive of a major public concern that violent video games lead to real-life violence," they wrote⁵.

Cartoon categories

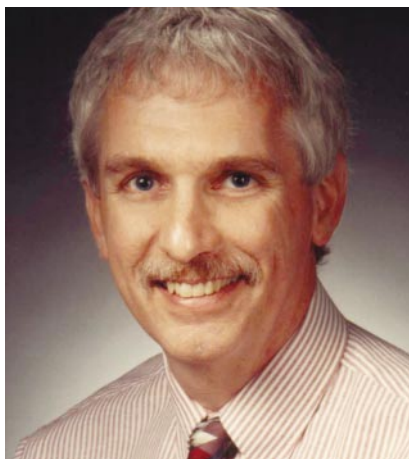
How can groups reach such different conclusions? Part of the answer lies in the difficulty of comparing studies. Take the definition of violent content, for example. One widely cited study of television violence was published in March by Rowell Huesmann, a psychologist at the University of Michigan. He found that subjects who had watched violent shows and identified with aggressive television characters as children showed more aggression in early adulthood⁶. But in the study, *Roadrunner* cartoons were described as "very violent", leading critics to question the method's validity. Assessments of video-game violence suffer similar problems. One group of researchers, for example, counted Pac Man being swallowed by ghosts as a violent event, and tallied 0.59 "deaths per minute" in a *Smurfs* game⁷.

Michael Rich, head of the Center on Media and Child Health at the Children's Hospital in Boston, Massachusetts, and a film-maker turned paediatrician, would like to end this confusion. "We need to get out of this mode where everybody's measuring different things," he says. The centre hopes to standardize existing studies of television and video games to allow direct comparison, and to develop a content-based ratings system grounded on the principles of developmental psychology.

Hints about how this could be done come from the work of Jeanne Funk, a psychologist at the University of Toledo in Ohio, who has helped to review rating standards for the software industry. Funk consulted with teenagers to come up with more subtle and descriptive categories, such as 'fantasy violence', 'human violence' and 'sports violence'. The Entertainment Software Rating Board, the New York-



Screen shot: Craig Anderson (below) suspects that the game *Doom* influenced Eric Harris and Dylan Klebold's rampage at Columbine High School.



based body that oversees US game classifications, plans to incorporate such categories in its labelling, and the descriptions could also help game-violence researchers to be more specific about what kind of content they are assessing.

Other problems may be more difficult to tackle. Different experiments often measure different proxies for aggression, for example. Some merely record signs of physiological arousal, such as increased heart rate and blood pressure, after subjects play violent games. Others try to assess violent thoughts, based, for example, on how subjects complete a partial story given to them. Few studies have looked at actual acts, such as blasting another person with sound in the lab, or hitting other children.

Although Anderson maintains that aggression in the lab is essentially the same as aggression in the real world, critics see the connection as tenuous. Both sides agree that carefully designed longitudinal studies — tracking the real-life histories of heavy game players — would advance our understanding.

But Anderson and his colleagues have been unable to obtain funding for such research.

Policy-makers, meanwhile, cannot wait for science to catch up — they come under pressure every time a violent new game is released, and have to act on the available evidence, even if it is limited. Almost every session of the US Congress includes an attack on the dangers that television and video-game violence pose to America's youth. And local law-makers are beginning to take action. In May, for example, Washington became the first state to ban sales of realistic cop-killer games to children younger than 17 years of age.

Small influence

Are such measures justified on scientific grounds? Some of the researchers consulted by *Nature* argue that computer games could lead to violent behaviour under certain conditions — they might trigger aggression in certain people already predisposed to violence, for example. But few support the idea that video games are important causes of violence in the real world. A 2001 report on violence issued by the US Surgeon General⁸ sums up their opinions: "Taken together, findings to date suggest that media violence has a relatively small impact on violence."

Some of the most damning verdicts on video-game studies come from those who study violence in general. Jeffrey Fagan, who heads the Center for Violence Research and Prevention at Columbia University in New York, says media-violence researchers are guilty of "a lot of sloppy thinking about causality". Look at it from an epidemiological viewpoint, he urges. With millions of copies of *Doom* sold, we would be seeing an epidemic of violence unless the dose-response rate is extremely small. Meanwhile, violence in the United States has declined — in 2001 it reached its lowest level since records began in 1972. Claiming that television or video games are important contributors to societal violence "doesn't pass the giggle test", says Fagan.

Anderson remains undaunted, however. While conceding that media exposure may not be the most important risk factor for violent behaviour, he insists that the link between video games and aggression "has pretty much been established". But he is likely to need more definitive evidence to persuade his colleagues, let alone the millions of kids eagerly awaiting the next action-packed game. ■

Tony Reichhardt writes for *Nature* from Washington.

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Military-funded research is not unethical

The key is to ensure that it is the military rather than the scientists who are regulated.

Sir — Of particular interest in your News Feature “Remote control” on the state of neuroengineering research (*Nature* **423**, 796–798; 2003) is the discussion of the ethical issues and views attributed to Professor Martha Farah, that any researcher who accepts funding from the US Department of Defense without agreeing with the goals of the programme compromises his or her ethics.

First, the goal of the programme (www.darpa.mil/dso/thrust/biosci/bim.htm) is the development of new computational and microsystem tools to study biological systems. It is hard to see how these goals pose ethical problems for researchers. However, looking beyond the officially stated goals of the programme to the broader issue of military funding, your News Feature says that some scientists feel using money from the military is ethically problematic. This view ignores the historical and social context of military research. If having a well-regulated US military is both necessary and ethically sound, given the

current state of the world, then there is no reason to believe that supporting this capacity in the form of basic research poses an ethical dilemma. The key is to ensure that it is the military who remain ‘well-regulated’ rather than the basic scientists.

The main argument against these ethical concerns, however, is the nature of knowledge itself, which has neither good nor evil attributes. Currently, the Defense Advanced Research Projects Agency (DARPA) is funding basic-science researchers in a non-classified capacity precisely because the principles on which a robust neural-control signal might be based are not yet known. Only the real-world application of such natural principles, once discovered, will enable good or evil to occur. In the case of neuroengineering research, the knowledge being generated clearly has the potential for a large positive impact on society. Decoding neural-control signals from paralysed or locked-in patients could allow them to regain physical function or the ability to communicate.

Many technologies funded by the US military have led to positive and even revolutionary changes in society. As a case in point, the Internet was originally conceived and funded by the previous incarnation of DARPA. The immense positive social impact of the Internet makes it clear that the presence of DARPA funding does not necessarily provide an accurate indication of how technology created from basic research will be used, and as such, has no relevance to ethical debate. The neuroengineering community serves the best interests of society quite well and we encourage a thorough and open debate of the applications built upon neuroengineering research.

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Although none of the authors is currently a direct recipient of DARPA grants, each has used DARPA funds in the past.

Military: brain machine could benefit millions

Sir — Your News Feature “Remote control” (*Nature* **423**, 796–798; 2003) misrepresented some aspects of the brain–machine interface programme funded by the US Defense Advanced Research Projects Agency (DARPA). The DARPA programme is basic research, defined by the Department of Defense as “systematic study directed toward greater knowledge or understanding of the fundamental aspects of phenomena and of observable facts without specific applications toward processes or products”.

DARPA recognizes that the development of new brain–machine interfaces will have restorative applications in the biomedical community. We embrace those advances as important technological goals for the future, and have funded projects like those you describe in your feature because of these key medical missions. We appreciate the tremendous benefit this technology will have to both defence personnel and civilians, especially the millions of military veterans who could profit from this work.

Your feature is inaccurate in describing how proposals were selected for the programme. Proposals were solicited from the research community in an open competition, and those funded were selected based on a review by a panel of experts. DARPA also does not as a practice

threaten insecurity. Our programmes are milestone-driven, as that is a way to ensure the best use of taxpayer dollars, but we are sensitive to working with academic institutions, and do our best to ensure stability in graduate education.

I also believe you have inaccurately advocated an ethical position in your Editorial “Silence of the Neuroengineers” (*Nature* **423**, 787; 2003). Although it is important to discuss the ethics of neuroscience research, the premise of your editorial and ethical argument is based on an inaccurate portrayal of DARPA funding. The reluctance of DARPA investigators to speak about the narrow vision of brain–machine interfaces that you portrayed relates to the basic-research nature of the DARPA funding rather than their ethics. The editorial states “Simply taking DARPA’s money, and citing possible medical benefits, is not enough”. Indeed, if these investigators succeed in their outstanding research, this should be more than enough. A sound ethical argument should also consider the opportunity lost for the millions of people who might benefit from important restorative benefits if the DARPA programme did not fund the work and the possibility of a new generation of brain–machine interfaces did not emerge.

Alan Rudolph

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Authorities should note sponsorship—results link

Sir — According to your survey of senior scientists in Germany (*Nature* **424**, 117; 2003), 80% feel that misconduct is a major problem in clinical research.

This outcome is consistent with a recent study published in the *Journal of the American Medical Association (JAMA)*, which reveals a significant association between industry sponsorship and pro-industry results (J. E. Bekelman, Y. Li & C. P. Gross, *J. Am. Med. Assoc.* **289**, 454–465; 2003). The JAMA analysis concludes that industry-sponsored studies are nearly four times more likely to reach pro-industry conclusions than are studies that are not industry-sponsored.

Obviously this is a major problem. The JAMA authors say “all investigators and sponsors undertaking human participant research should not only fully disclose the nature and extent of their relationships but also make available all research results from completed clinical trials in a comprehensive, publicly accessible registry”.

In my view, it should be mandatory for health authorities to consider the potential influence of industry sponsorship on the results of clinical studies during the approval process.

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Uncertainty rules

Neuroeconomics seeks to move brain studies beyond the fixed reflex.

Decisions, Uncertainty, and the Brain: The Science of Neuroeconomics

by Paul W. Glimcher

MIT Press: 2003. 375 pp. \$37.95, £25.50

P. Read Montague

"Doubt is not a pleasant condition, but certainty is an absurd one."

Voltaire

Uncertainty. The mere sound of the word elicits a shaky feeling, and in Voltaire's case, an unpleasant one. Uncertainty makes investments risky and decisions difficult, and, for better or worse, it spices most experiences. Engineers try to control it, financiers try to reduce it, and it haunts most of our everyday decisions. According to Paul Glimcher, we should embrace uncertainty, model it and use it to connect our growing knowledge of brain function to cognition. And from where will the new neural models of uncertainty arise? Economics. This premise is enticing, but the scientific issues in this book require a lot of unpacking. Who is Paul Glimcher and what is he saying?

A talented neuroscientist at New York University, Glimcher has produced the first full-length book in the fledgling field of neuroeconomics. This nascent area, which has already attracted several economists and neuroscientists, assumes that the brain generates economic behaviour; consequently, economics can benefit from neuroscience. The benefits can also flow in the reverse direction: economic theory can be used to frame, model and understand neuroscience experiments. Glimcher is not just enthusiastic about this latter direction, he's downright evangelistic. To fully understand the nature of his fervour, one has to tackle the entire book.

Glimcher's enthusiasm derives from a conceptual problem that he thinks has plagued most neuroscience research since the time of René Descartes. Glimcher argues (across four chapters) that Descartes' legacy is an implicit model, shared by most neuroscientists, in which the reflex is the underlying mechanism from which more complicated functions such as decision-making are constructed.

We all know about reflexes. If you knock the knee with a rubber mallet, signals return to the spinal cord, relay to output neurons there, and cause an extension of the lower limb. Hold a candle to an outstretched hand and the hand withdraws, again a simple reflex. Glimcher's claim is that this idea of



Crash course: what can the 1929 New York stock-market collapse teach us about the brain?

the reflex ossified into a neural prescription for the way in which all complex behaviours are constructed. According to Glimcher, the idea of the reflex provides "a simple set of basic operations that could be combined in different ways to yield a working model for any determinate behavior". He calls this idea 'reflexology'. Most readers will probably assume that Descartes and maybe some of the more strident behaviourists are the only reflexologists. Wrong, says Glimcher — the vast majority of neuroscientists are basically reflexologists in this sense. And so too are all the neural-network people. Yikes! And what's worse, we really aren't aware of our problem. The case that Glimcher builds has many parallel streams and can be difficult to follow. Let's continue to unpack his ideas.

Glimcher thinks that neuroscience is in this state because we wrongly view 'reflex thinking' merely as a way of collecting data about simple nervous systems or small isolated parts of complex nervous systems. Instead, says Glimcher, reflexology is really a theory, or a philosophical stance on how to break neural function into its component pieces, and it is woefully inadequate. But there is a solution: computational goals and economic analysis.

According to Glimcher, the computer scientist and neural theorist David Marr was the first to propose a way out of the murky waters of reflexology. Glimcher praises

Marr's focus on the types of computational problem that the nervous system is trying to solve. He quotes Marr: "An algorithm is likely to be understood more readily by understanding the nature of the problem being solved than by examining the mechanism (and the hardware) in which it is embodied."

With that development in hand, Glimcher defines the objective of all behaviour: "The goal of the nervous system, ultimately, must be to produce motor responses that yield the highest possible inclusive fitness for an organism." The idea is that the invisible hand of fitness has long sculpted the way that neural systems deal with an uncertain world, and that we should expect to find neural modules dedicated to dealing with uncertainty. As Glimcher explains over five chapters, these modules are best examined using economically framed theories.

The next section of the book is the most engaging as the author has personalized the descriptions. These chapters cover a series of experiments in which behaviour and neural activity were measured concurrently to discover how neural activity relates to the optimal and suboptimal behaviour expressed by the experimental animals. Glimcher also delivers a short primer on game theory and its applications to behavioural ecology and neuroscience.

The most interesting discussion point is Glimcher's assertion that there are two types

AP PHOTO

of uncertainty: reducible and irreducible. The former is uncertainty about the external world, and can be reduced through exploration and learning. Only limited resources prevent this type of uncertainty from being completely eliminated.

Irreducible uncertainty, on the other hand, is more interesting, especially for its effect on behaviour. As Glimcher points out, Werner Heisenberg's uncertainty principle makes clear that the physical world has a quantifiable level of irreducible uncertainty that no amount of measurement or knowledge can eliminate. Glimcher extends this claim to the behavioural realm using game theory. The basic idea is that organisms will express irreducible uncertainty in their behaviour. The driving constraint that selected for such irreducible uncertainty is the need for interacting organisms to outperform others with whom they compete or on whom they prey. If organisms had only very complicated strategies for behaviour, it is conceivable that over millions of years a competitor could adapt to those strategies. Irreducible uncertainty, as a component of a behavioural strategy, cannot be learned and exploited by an opponent. Glimcher offers several concrete examples of these ideas. One take-home message is that most complex creatures should have internal processes that are roughly equivalent to a random-number generator.

Glimcher's central programme outlined in the book is laudable: determine exactly what a particular behaviour is 'for', quantify it in formal economic terms, and design experiments around the formal model. As he freely admits, this formula, although beguiling, is easy to state but hard to carry out. It reminds me of the maxim of novelist W. Somerset Maugham: "There are three rules for writing the novel. Unfortunately, no one knows what they are." Although it's not quite that bad in neuroscience, some features such as conscious awareness are difficult to understand from a computational perspective.

On balance, the book is provocative, encouraging a serious reconsideration of the utility of most neurophysiological work in alert animals. Is it really true that all such efforts are hamstrung by the implicit bogeyman of reflex theory? In what sense do present-day neurophysiologists make implicit assumptions consistent with such a limited view? Glimcher's claim in this area will certainly raise the hackles of more than a few, but what good is a book if it does not provoke? This book will surely ignite discussion and soul searching among serious neuroscientists, and Glimcher has bravely offered us a clear model to talk about. ■

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Beauty and the bees

Form and Function in the Honey Bee

by Lesley Goodman

International Bee Research Association

(www.ibra.org.uk): 2003. 220 pp. £25 (pbk), £55 (hbk)

Thomas D. Seeley

The best-studied of all the millions of insect species in the world is the honeybee, *Apis mellifera*, particularly with regard to its sensory physiology and functional morphology. Since the pioneering studies of Karl von Frisch and Robert E. Snodgrass in the early part of the twentieth century, several generations of biologists have carefully measured the powers of discrimination of worker bees in every known sensory modality, analysed the mechanisms underlying these abilities with behavioural and electrophysiological techniques, and used light and electron microscopy to explain the anatomical bases of the bee's behaviour. As a result, the honeybee provides a solid baseline for comparative studies of most aspects of insect behaviour, physiology and morphology.

The composite picture assembled from all this work is one of highly developed sensory capacities and motor performances. Honeybees see the world in colour, perceive shapes and patterns, and can resolve rapid movement. Their olfactory sense is almost identical to ours, and their sense of taste is similar but generally less sensitive. Mechanosensory perception — including touch and sensitivity to airborne and substrate-borne vibrations — is also extremely rich as the bees have thousands of sensory hairs all over the body (even on the compound eyes) and stretch receptors inside the body, giving information on position, movement and orientation relative to gravity. Honeybees even have at least a limited responsiveness to Earth's magnetic field.

These impressive sensory abilities are used for sophisticated manipulatory behaviours such as building beeswax combs and negotiating complicated flowers, flying over distances of several kilometres to reach flower patches rich with nectar and pollen, and communicating with hive-mates by means of diverse shakings, tappings and buzzings, and puffs of chemicals.

Form and Function in Honeybees by the late Lesley Goodman is a modern synthesis of honeybee sensory physiology and functional morphology. The last attempt at a comprehensive treatment of the sensory basis of this insect's behaviour was von Frisch's classic *The Dance Language and Orientation of Bees*, published in 1967. Not only has the literature on the subject increased enormously since then, but there is now a greater sophistication in understanding the ecological significance



Touchy-feely: the antennae and hairs of honeybees are equivalent to human fingertips.

of each sensory ability. For example, it is now known how the bee's colour vision system, which renders bees maximally sensitive to differences in light at wavelengths of about 400 nm (violet) and 500 nm (blue), has fostered the evolution of flowers with pigment combinations that have sharp rises and falls in reflectance in these two regions; such combinations are most easily discriminated and recognized by the bees. There can be no doubt that this book addresses an important need — and meets it beautifully.

Goodman started planning this book in 1996 with the ambitious goal of producing a volume on how bees function that would be both scientifically rigorous and yet readable (and also affordable) to a broad audience of beekeepers, undergraduate biologists and research scientists. She was unable to finish this project before her death from lung cancer in 1998, but did set up the L. J. Goodman Insect Physiology Research Trust to ensure that the book was completed posthumously. Thanks to the dedicated work of Richard J. Cooter, one of her first PhD students, and Pamela Munn, deputy director of the International Bee Research Association, this final wish was fulfilled magnificently.

The book is utterly gorgeous: each page is lavishly illustrated with beautiful coloured diagrams, specially commissioned paintings

and superb micrographs. Such a beautifully crafted book is rarely seen in science. Given its large format (24 cm × 34 cm) and its opulent contents, it can be valued as much as a work of visual art as an informative work of biological science. But I wish to emphasize that this is a rigorous scientific monograph. The contents are well referenced to the scientific literature (up to the mid-1990s), and the writing is clear and crisp.

It provides a wide audience with an astoundingly beautiful and admirably accurate account of how a worker honeybee smells and sees, tastes and touches, feeds and breathes, flies and stings, secretes wax and releases pheromones. Goodman has given all who are interested in the behaviour and physiology of bees an amazing gift. ■

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More on bees

Bumblebees: Behaviour and Ecology

by Dave Goulson

Oxford University Press, £55 (hbk), £27.50 (pbk)

Universal support

Echo of the Big Bang

by Michael Lemonick

Princeton University Press: 2003. 232 pp.

\$24.95, £17.95

Sean Carroll

The first pages of Michael Lemonick's new book grab the attention. Princeton University astrophysicist David Spergel is fretting over the implications of a new discovery, made from observations of the cosmic microwave background (CMB), which according to Lemonick implies that "much of the work in cosmology over the past two decades has been based on a faulty theoretical foundation". The observations are from NASA's Wilkinson Microwave Anisotropy Probe (WMAP), a satellite that measures, with unprecedented precision, temperature fluctuations in the relic radiation from the Big Bang.

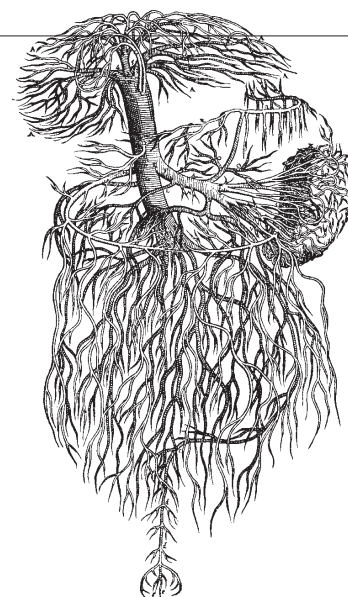
It is a gripping vignette, but puzzling: as we now know, the WMAP results didn't include any major surprises. If anything, WMAP provided a convincing capstone to a series of impressive advances in cosmology, demonstrating that our consensus picture of the Universe is fairly accurate. This picture can be hard to swallow, as it describes a Universe of which only 4% is made up of ordinary matter; the rest is a mixture of cold dark matter and an exotic 'dark energy', which remains nearly constant throughout space and time. When a purported consensus leans so heavily on the invocation of unseen substances, it is important to verify it from every

Vesalius's vessels

When Andreas Vesalius published his *De Humani Corporis Fabrica Libri Septem* (*On the Fabric of the Human Body*) in 1543, its accurate depiction of the human body from close observation of dissected bodies overturned centuries of medical dogma and led the way for the modern study of anatomy. William Richardson and John Carman have been translating this treatise; the third volume, containing Books III and IV from the original work, focusing on the veins, arteries and nerves, has now been published (Jeremy Norman, \$250). Although the accuracy of Vesalius' work on these soft tissues has not stood the test of time as well as the earlier skeletal work, the illustrations, like that of the portal vein shown here, are stunning. The vessels appear as if fixed in space, with the other internal organs simply invisible.

www.historyofscience.com

Mary Purton



angle; the WMAP results were justly hailed as an impressive example of such a verification.

Of course, the WMAP team did make fresh discoveries, as well as confirming existing views. The satellite measured the polarization of the CMB on large angular scales, which can be interpreted as evidence for re-ionization of the Universe by early star formation. In addition, a few tantalizing features of the WMAP data can be interpreted as evidence for something new, but these are hints rather than firm results; nobody has been moved to overthrow the theoretical foundations of cosmology.

The opening chapter does not explain the nature of the unsettling discovery to which it refers; it is a teaser, meant to draw the reader into the rest of the narrative. The mystery is only revealed near the end, where we learn that it refers to the possibility that the data indicate a finite spatial size to the Universe. But we also learn that this possibility is not taken very seriously; Spergel is quoted as saying: "We don't think it means anything." And Lemonick concludes the book by admitting that the WMAP team "wouldn't be saying anything shocking". A reader might feel somewhat cheated at this point.

This episode is emblematic of the frustrating nature of the book. Lemonick was given access to the inner workings of the WMAP team (as an embedded journalist, if you will), a collaboration whose deliberations were closely guarded from the rest of the scientific community. But Lemonick seems to want to tell a slightly different story to the one that actually happened — one in which a heroic band of plucky scientists raced against outside competition and fought valiantly against recalcitrant NASA bureaucracy, succeeding in the end in redrawing our picture of the Universe. In

reality, the talented and dedicated WMAP team worked within the context of numerous experiments, taking complementary approaches to understanding cosmology. The result was a triumph of the congratulatory rather than the revolutionary kind: we had it right all along.

The insider's view of how real scientists go about their everyday business is one of this book's strongest points. The human element is well illuminated, as in the account of the excitement and pressure accompanying the satellite launch itself. Indeed, human concerns are perhaps emphasized too much; the struggle over whether satellite components were to be assembled on Princeton's campus or at a NASA centre is likely to be less fascinating to the general reader than it was to the participants at the time.

A more important complaint is that the book takes too many shortcuts in explaining the science behind the CMB observations. It is hard to understand, for example, why the most visually striking product of the WMAP observations — a high-resolution map of the CMB fluctuations — is not included. For some reason, a simulation of the map is reproduced, but not the map itself.

WMAP (named after David Wilkinson, a team member and CMB pioneer who died in 2002) marks the end of a decade of upheaval and triumph in cosmology, which began with the original discovery in 1992 of CMB fluctuations by COBE, another NASA satellite. This book describes a small but important part of the achievements of the past 11 years. A comprehensive view of this most exciting time in the history of cosmology is a worthy story that remains to be told. ■

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A genetic melting-pot

Marcus W. Feldman, Richard C. Lewontin and Mary-Claire King

Race as a biological concept has had a variety of meanings. In the taxonomic literature, a race is any distinguishable type within a species, such as dark-bellied and light-bellied variants of small mammals. In 1937, Theodosius Dobzhansky introduced the idea of geographical races — populations of species that differ in the frequencies of one or more genetic variants. But as no two populations have identical gene frequencies at variable (polymorphic) loci, Dobzhansky's definition of race becomes synonymous with that of population.

The classical definition of race, as applied to our species, is based on phenotypes such as skin colour, facial features and hair form that clearly differ between native inhabitants of different regions of the world. An underlying assumption is that all of these defining features (all largely genetic traits, although few of their genes have been identified) are characteristic of the genome in general. In other words, just as there are large differences between races in genes for skin colour, so there should be large genetic differences between races in general. In the previous absence of data to confirm or deny this assumption, it was not an unreasonable one to make.

But recent studies of genetic diversity indicate that the genes underlying the phenotypic differences used to assign race categories are atypical, in that they vary between races much more than genes in general. Together, the iconic features of race correlate well with continent of origin but do not reflect genome-wide differences between groups.

Discussion has arisen over the implications of these findings for the utility of racial classification in medical practice. The issue of whether race is a biologically useful or even meaningful concept when applied to humans in a medical context is controversial — holders of opposing views each claim to have evidence to support them. But there is no contradiction between these two well-substantiated bodies of data, as they actually

deal with two different questions that have become confused with one another.

The first question is: "Is it possible to find DNA sequences that differ sufficiently between populations to allow correct assignment of major geographical origin with high probability?" The answer to this question is yes, as shown by studies of genetic polymorphisms and by universal personal experience.

The second question is: "What fraction of human genetic variation, whether based on protein-coding genes or other sequences, falls within geographically separated populations, and what fraction occurs between these populations?" The answer to this question is that most genetic diversity occurs within groups, and that very little is found between them.

Why this apparent paradox? The answer is that genes that are geographically distinctive in their frequencies are not typical of the human genome in general.

It has been suggested that racial categorization has a valid role in good medical practice because many medically important genes vary between populations from different regions. But although knowing a patient's ancestry is often extremely useful in diagnosis and treatment, race is both too broad and too narrow a definition of ancestry to be biologically useful.

For any species, definitions of race can lose their discriminating power when individuals migrate to different regions and mate with their counterparts there. Among humans, large-scale migrations between continents — particularly through European colonial expansion and the commercial slave trade — has resulted in matings of individuals from different continents and the creation of new populations, especially in the Western Hemisphere and Oceania. Many people thus have ancestry from more than one major geographical region, meaning that the association of phenotype and geography breaks down.

For example, sickle-cell disease, which is often thought to be an African trait, is instead characteristic of ancient ancestry in a geographic region where malaria was endemic. Africa is one such region, but so are the Mediterranean and southern India. If sickle-cell disease is suspected, then the correct diagnostic approach is not simply to determine the patient's race, but to ask whether they have African, Mediterranean or South Indian ancestry. To use genotype effectively in making diagnostic and therapeutic decisions, it is not race that is relevant, but both intra- and trans-continental contributions to a person's ancestry.

Race and ancestry are confounded both by genetic heterogeneity within groups and by the widespread mixing of previously iso-

Race

Ancestral genetic data are far more useful for medical purposes than are racial categories, which may be correlated with disease for social or economic rather than biological reasons.

lated populations. The assignment of a racial classification to an individual hides the biological information that is needed for intelligent therapeutic and diagnostic decisions. A person classified as 'black' or 'Hispanic' by social convention could have any mixture of ancestries, as defined by continent of origin. Confusing race and ancestry could be potentially devastating for medical practice.

Other attempts to classify people into broad genetic groups based on the frequency of specific genes for, say, drug-metabolizing enzymes, are also likely to be poor predictors of medical outcome. As with racial groupings, the overall variation in the frequencies of such genes between groups is likely to be less than that within each group.

The conventional, social definition of race is useful in a medical context as it provides information about the social circumstances and lifestyle of patients. But this is a consequence of social history, so any variation is (at least in principle) transitory. By contrast, information on the likelihood that a person carries specific disease-related or treatment-response genes is grounded in their ancestry in far more complex ways. We suggest that identifying all contributions to a patient's ancestry can be useful in diagnosing and treating diseases with genetic influences. Eventually, for both diagnosis and treatment, specific genetic variants will provide concrete, useful information.

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Human migration, as depicted in Charles Hunt's *Landing at Madras*, blurs the boundaries of race.

A tale of tails

Christof Niehrs

Developing organisms still hold many surprises for biologists. For instance, an entirely new 'organizer' — a clump of cells that tells other cells what to do — has been discovered at a very early stage of zebrafish development.

The vertebrate tail is a somewhat neglected organ. Protruding from the rear, it does not, at first glance, seem to serve much purpose other than to chase off flies on cows and horses. In humans, the rare atavistic occurrence of a rudimentary tail can even be an embarrassment. Yet the post-anal tail is one of the defining features of chordates. In primitive chordates and fish it promotes locomotion; lancelets use it to burrow in sand; in mammals it stabilizes posture and allows communication; and monkeys also use it as a third leg. All vertebrates develop a tail as embryos, and where the adults do without, it disappears during fetal development or metamorphosis. Despite its importance, however, we know little about the molecular mechanisms that underlie tail formation. On page 448 of this issue, Agathon *et al.*¹ present an analysis of tail development in zebrafish. Unexpectedly, they have discovered a new 'tail-organizing centre' in early embryos, and have shown that its molecular components include some of the usual suspects.

The question of how the embryonic tail forms is part of the greater problem of how the main body axis — from head to trunk to tail — is generated during early vertebrate development. Of central importance for the establishment of this body axis in amphibians is the upper dorsal blastopore lip, better known as the Spemann organizer, which can, after being transplanted into a different embryo, cause a second body axis and hence a twin embryo to form. Structures equivalent to the amphibian organizer have been found in all other vertebrates. The organizer produces signals that control the specialization of nearby tissues; two of its main functions are to induce the production and patterning of neural tissue, and to cause ventral tissues of the middle embryonic layer known as the mesoderm to form dorsal tissues (such as muscle)².

Early transplantation experiments by Otto Mangold³ showed that the Spemann organizer can be subdivided into head, trunk and tail organizers (Fig. 1a). So it comes as a complete surprise that Agathon *et al.*¹ have discovered a tail-organizing centre that appears to be independent of the Spemann organizer. The authors carried out experiments with zebrafish blastulae, early embryos that consist of just a hollow ball of cells. They took cells from the ventral margin

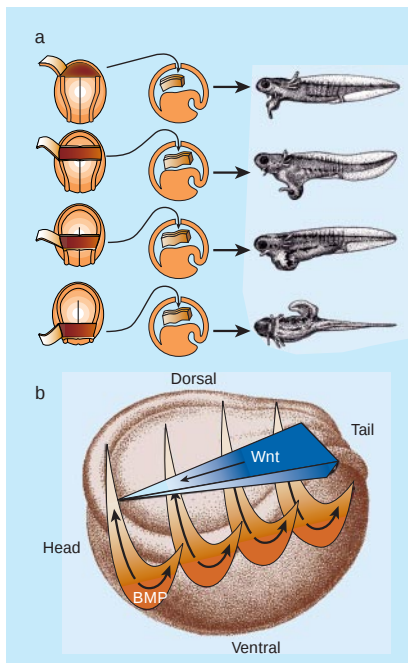


Figure 1 Organizers of development. a, The Spemann organizer. Otto Mangold³ took different segments from the Spemann organizer of early newt embryos (neurula stage; left), and transplanted them into the fluid-filled cavity of embryos at the even earlier gastrula stage (centre). As tadpoles (right), the embryos displayed duplicate, region-specific balancers (stabilizing threads in the head, heads, trunks or tails). (Modified from ref. 10.) b, Double-gradient model for generation of the body axes, showing how perpendicular activity gradients of Wnts and bone morphogenetic proteins (BMPs) regulate head-to-tail and dorsal-ventral patterning. The gradients are indicated by colour scales; arrows indicate spreading of the signals. Patterning begins at gastrula stages, but for clarity is depicted in an early amphibian neurula. The formation of head, trunk and tail requires increasing Wnt activity. Agathon *et al.*¹ have found that tails develop where high levels of BMP and Wnt signals intersect, towards the posterior. (The model is adapted from ref. 9 and is a molecular interpretation of the classical double-gradient theories reviewed in ref. 11.)

— a tissue that does not become part of the 'shield', considered to be the fish equivalent of the Spemann organizer — and transplanted the cells into host embryos. The result was extra tails, consisting of donor- as well as host-derived cells. Agathon *et al.* also showed that inactivating the Spemann organizer did not interfere with this 'ectopic' tail formation, implying that the two organizers are indeed independent. The authors concede, however, that the Spemann organizer is required for 'proper' tail formation, as the ectopic tails were incomplete: for instance, they lacked the stiff supporting rod known as the notochord, which is derived from the Spemann organizer.

The authors also established that this tail-organizer activity involved growth-factor proteins of the Wnt, bone morphogenetic protein (BMP) and Nodal families. These signalling molecules were good candidates for such a role because they are known to be present in the ventral margin, and interfering with the signalling pathways to which they contribute inhibits tail formation. Agathon *et al.* now show that misexpressing these growth factors in combinations, but not singly, leads to the generation of extra tails — which are, again, incomplete. So all three growth factors are involved in

the newly discovered tail-organizer activity.

The wider significance of these findings concerns how they relate to existing models for region-specific organizers, in which Wnts, BMPs and Nodals are no strangers. The head- and trunk-organizing activities of the Spemann organizer are known to require combinations of these three types of growth factor⁴. Head-organizer activity requires inhibition of all three signals; trunk formation requires Nodal signalling to be active but BMP signalling to be inhibited; and we now know that the tail organizer requires signalling by all three factors. The drawback of previous organizer models was that they failed to explain the difference between the trunk and tail organizers in molecular terms, except for tail development at later stages^{5,6}. Thanks to Agathon *et al.*, this riddle seems well on the way to being solved.

A few issues remain, however. First, Agathon and colleagues' model¹ emphasizes qualitative differences in combinatorial signalling, with key signals being either 'on' or 'off' — and this is enough to explain the phenomenon of discrete organizers as they have been observed in transplantation experiments for the past 75 years. For instance, the authors propose that Wnt signalling needs to be 'on' to generate the tail,

but 'off' to produce the trunk. Yet we know that all three growth factors act in a concentration-dependent fashion during early axis formation. A gradient of Nodal protein instructs cells at different points along the gradient to take on different mesodermal fates; this then translates into an increasing dorsal-to-ventral gradient of BMP^{7,8} and an increasing head-to-tail gradient of Wnt⁹, generating a continuum of positional information. Completely blocking Wnt inhibits trunk formation, so trunk-organizer activity must require some — probably low levels of — Wnt signalling, rather than the complete inhibition suggested by the authors.

Second, unlike BMPs and Wnts, Nodal proteins affect the head-to-tail patterning of neural tissue only indirectly, inducing mesendodermal tissue to produce, for instance, Wnts, Wnt inhibitors and BMP inhibitors at different threshold activities of Nodal. A model complementary to that of Agathon *et al.* focuses on the more direct players — BMP and Wnt — in regulating axis formation (Fig. 1b), and takes into account their activity gradients.

Particle physics

Strange days

Frank Close

Three new subatomic particles have been found, and all survive for an unusually long time before they decay. Physicists now face the challenge of explaining this within the framework of the existing theory.

“It is as if Cleopatra had fallen from her barge in BC and had not yet hit the water.” Such was the description half a century ago of the discovery of the astonishingly long lifetime (up to about 10^{-8} seconds) of strange particles. Everyday matter, such as protons and neutrons, is made of two types of quark, known as 'up' and 'down' (Fig. 1). But in 1947, new particles were discovered that contained a third type of quark, called 'strange'¹. Today, strange particles are a well-established part of the standard model of particle physics, which now includes six types of quark. We know that their seemingly long lifetimes are a consequence of the 'weak' interaction that they undergo in decaying; if instead they were subject to the powerful 'strong' interaction their lives would be over in around 10^{-23} seconds. Instead, death by decay is neutered by the presence of strangeness.

In the past two months, three different particles have been discovered, and explaining them has proved a challenge for theorists. Although not as extreme as the above example, each of these new particles has an unusually elongated lifetime. Two of them are 'mesons', each containing a strange anti-quark and a charm quark (the fourth quark

Finally, how does the tail organizer uncovered by Agathon *et al.* interact with the Spemann organizer to induce complete tails? And how can we integrate the process of trunk 'segmentation', which is controlled from the tail bud? Whatever the answers, the new work¹ should generate greater interest in questions relating to the tail.

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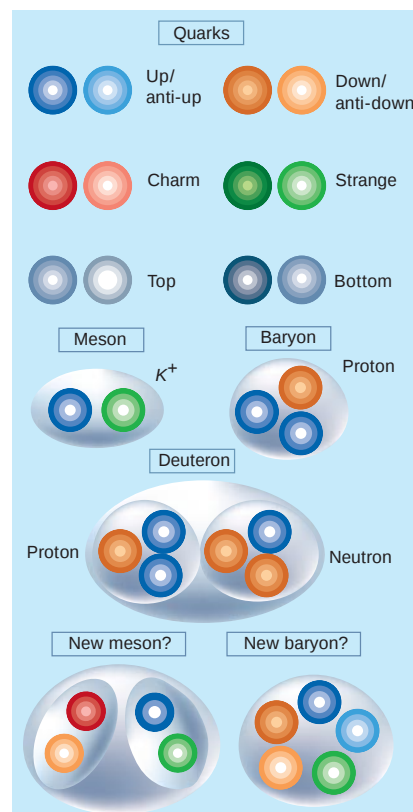


Figure 1 Quarks and particles. In the standard model of particle physics, there are six quarks — fundamental particles that are the building-blocks of many others. Each quark also has an anti-matter partner, an anti-quark. Pairings of quarks and anti-quarks form 'mesons', such as the K^+ ; three quarks form 'baryons', such as the proton. The picture builds up further: a three-quark proton and a three-quark neutron together form a deuteron; adding more protons and neutrons — more three-quark combinations — builds up atomic nuclei. The discoveries of what seem to be a new meson^{2,3} and a new baryon⁴ don't easily fit the established picture. The new meson may in fact be a 'molecule' of two mesons, and the baryon might be a 'pentaquark' state.

to be formed from just three quarks. In addition, QCD seems to allow more complicated clusters of quarks or anti-quarks — atomic nuclei are familiar examples of quarks bound in multiples of three. An open question is whether there are analogues containing anti-quarks. The simplest would be two quarks balanced by two anti-quarks, in effect a 'molecule' of two conventional mesons, or three quarks accompanied by an additional quark and an anti-quark, making a pentaquark.

Unambiguous evidence for such states in the data is lacking. Their absence is attributed to the ease with which they would fall apart into a pair of conventional mesons, or a meson and a baryon. It is estimated that they would survive for less than 10^{-24} seconds, which is at the current limit of detection. But the sightings of the three metastable

particles, reported by the BaBar² and CLEO³ experiments in the United States and the SPring-8 experiment⁴ in Japan, may at last be proof of these states' existence.

The baryon is utterly novel. In 60 years of studying strange particles, no such combination of electrical charge and strangeness (one positive unit of each) with baryon nature has been seen. The original sighting in Japan has been corroborated by two other experiments^{5,6}, but all of the detections are at levels that are currently on the borderline of significance, limited by the amount of data available. The simplest response is to wish it away. But within the next year a high-statistics experiment is planned to establish whether it really exists and, if so, to measure its properties (such as its spin). If it is real, then it may be most naturally explained as a pentaquark, containing a strange anti-quark. Its metastability would require that it is one of a family of particles related through a property called 'isospin'. Because isospin must be conserved (in the same way that, for example, energy and momentum must be conserved when billiard balls collide), the number of ways in which these particles can decay is restricted, and so they cannot decay quickly. If this picture is correct, it implies the existence of more of these baryons with unusual correlations of charge and strangeness, and they could be searched for in moderately high-energy experiments.

By contrast, there is no doubt about the existence of the two meson states, both known as 'D_s'. They appear as clear peaks in the data and their spins almost certainly have the values zero and one (thus they are referred to as 'scalar' and 'axial' mesons, respectively). They have all the characteristics of states made from a charm quark and a strange anti-quark, but, for some reason,

have masses that are lower than expected — so much lower, in fact, that their natural decay paths (into a charm meson and a strange meson) are energetically closed. This is the cause of their metastability. But the question of why they are so much lighter than their siblings in the charm–strange family is still to be resolved.

One possibility is that they are better described as 'molecules' — bound states of mesons, one containing a charm quark and the other a strange quark, with energies slightly below the fall-apart threshold. This is analogous to a proton and a neutron binding together to form a deuteron, and such behaviour has been seen elsewhere for scalar (spin zero) mesons. The masses of the newly discovered mesons are tantalizingly close to the thresholds for some two-meson states (in the case of the scalar meson, a molecule of a *K* and a *D* meson; and in the case of the axial meson, of a *K* and a *D*^{*} meson). It seems certain that these meson combinations play some role in lowering the observed masses of these new-found particles.

Further ways of producing these enigmatic states, along with more precise measurement of their properties, are now being pursued to identify the sources of their unexpected longevity. ■

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Neurobiology

Caught in the act

Yadin Dudai

Researchers may have seen the signature of a memory in the making. In monkeys that learnt to associate two stimuli, single neurons changed their responses before, during or after learning became evident.

When Captain Lemuel Gulliver visited Balnibarbi, he was fortunate enough to be allowed into the hall of Speculative Learning at the Academy of Lagado, where, with great admiration, he watched how the nuts and bolts of a knowledge machine generated new sentences of great wisdom in real time¹. Neurobiologists can only envy him to this day. How nice it would be to watch biological learning machines in action, and see how experience is converted into a memory trace. In the real world, this is more easily wished for than

done. Writing in *Science*, however, Wirth and colleagues² have moved a step closer to this goal. Their report is of particular interest because it gives fresh insight into the activity of the hippocampus — part of the brain machinery that forms and stores declarative (consciously accessible) memories, and a focus of attention for experimenters, clinicians and theoreticians alike³.

Wirth *et al.*² trained their monkeys in an instrumental conditioning task, in which the subject learns, by trial-and-error, the impact of its actions on the world⁴. Briefly, the

monkey was placed in front of a computer screen, and each trial started with the animal fixating on a central spot for 0.3 seconds. The monkey, continuing to fixate, was then presented with four identical visual targets simultaneously, superimposed for half a second on a complex, coloured visual scene. The background scene, but not the four targets, then disappeared, and after a delay of 0.7 seconds the fixation spot also disappeared, cueing the animal to move its eyes towards one of the targets. Only one of these targets was associated with a reward — a squirt of fruit juice. An experienced monkey required about a dozen trials to learn the correct position of the rewarded target in each new scene. On their road to fame, the two monkeys involved in this study saw a total of 378 new scenes over the course of 18 months, of which they learned 290 as required.

And as the monkeys' eye muscles worked for the juice, so too did the neurons in their hippocampus. Wirth *et al.* recorded the electrical activity of these neurons in order to get a handle on their role in learning. This was made feasible by the fact that the associative task is, on the one hand, not too easy, so that it takes a monkey some time to master it — enough time for the experimenter to follow neuronal dynamics. On the other hand, however, the task is not too difficult, meaning that the monkey can still succeed while the experimenter 'holds' a given nerve cell (in practice, not more than 30 to 50 minutes). So this is an example of cross-level analysis, tapping into behavioural and cellular processes concurrently — virtually essential for studies that aim to analyse the biology of memory.

Wirth *et al.*² recorded from 145 neurons in total, and found that 89 responded in a scene-specific manner at one or another phase of the session; of these, 25 changed their activity in close association with the animal's behavioural learning curve. It is this latter subset, dubbed 'changing cells', that attracted the authors' attention. Each of the 25 neurons showed robust changes in firing rate at some point following the presentation of a new scene, but little or no response to a highly familiar scene.

Furthermore, these changing cells fell into two categories. One category consisted of neurons that showed little or no response when a new background scene was presented or during the delay period of the task. But they then signalled that the animal had learned the location of the reward-associated target in that scene by significantly increasing or decreasing their firing rate. This altered activity was maintained throughout the recording session, suggesting that the cells were engaged in storing memories, or in monitoring their storage. The second category comprised neurons that responded to new scenes by either increasing or decreasing their activity relative to the baseline, and then signalled learning by returning to baseline

firing rates. Using analytical tools developed in modelling the response of cortical neurons⁵, Wirth *et al.* determined that both types of change reflected a modification of the selectivity of the neuronal response to learned stimuli compared with new stimuli.

The authors also compared the number of the trial in the recording session (trial 1, trial 2, and so on) in which behaviourally detected learning occurred, with the number of the trial in which neural activity changed. The results showed that the change took place in individual cells before, at the same time as, or after behavioural learning became evident. Wirth *et al.* took this to mean that hippocampal nerve cells that subserve the new associative memory were recruited into action progressively.

The hippocampus is part of the mediotemporal lobe of the brain, and Wirth and colleagues' study is not the first to report experience-dependent alterations in this overall region as a consequence of associative learning. For example, such phenomena have been seen in the perirhinal cortex^{6,7}. What distinguishes the new study² is the focus on the hippocampus, combined with the improved ability to monitor, in real time, the changes in activity of individual hippocampal neurons in correlation with the development of the learned behavioural response.

Aficionados of the hippocampus, learning, or both, might be tempted by the new data to pose additional questions. For example, predominant theories of learning consider the hippocampus and neocortex to be complementary memory systems that together subserve the acquisition and consolidation of long-term memories⁸. So, what is the relationship between the changes seen in the hippocampus and the learning-correlated changes that have previously been observed in neocortical neurons^{9,10}?

And how do we interpret the observation that the neuronal changes occurred at different time points relative to the emergence of behavioural learning? Does this, as Wirth *et al.* suggest, reflect an incremental learning process — a continuous time- and experience-dependent recruitment of pieces of the memory puzzle — which must cross a certain quantitative threshold to become behaviourally noticeable? Or are we on the trail of qualitatively different phases in learning, such as the encoding of 'online' information versus its registration as a new memory⁴? Do the cells indeed store the new memory, or instead only take notice of, or index, memories stored elsewhere? And what are the types of nerve cells involved and the modulatory inputs they receive from other brain areas?

These and other questions notwithstanding, this study² reflects the fact that we are at an interesting stage in memory research. The paper epitomizes our ability to eavesdrop on

neuronal conversations in the brain. Alas, no matter how skilfully the antennas are operated, without deciphering the semantics of the language we are unlikely to come up with definitive answers to some of the key questions. Yet the approach itself is also an attempt to produce a dictionary of neuronal language, and may in due course generate the tools it needs to succeed. ■

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Materials science

On the straight and narrow

Richard A. Register

Nanoscale chemical patterns written on a substrate can direct the self-assembly of polymer overlayers with remarkable precision. These polymer films, in turn, can be used as templates for nanofabrication.

Nanostructures of precisely defined size and position are essential for any addressable nanoscale device — such as an ultrahigh-density hard drive, in which each bit of material stores a bit of information. Amphiphilic organic molecules are attractive templates for making such structures, as they spontaneously form supramolecular aggregates (such as micelles) of near-uniform size, generating a multitude of identical nanoscopic objects in parallel. But this same feature causes a problem: the aggregates form simultaneously in many uncorrelated locations and so the nanostructures are never precisely positioned. On page 411 of this issue, Kim and co-workers¹ overcome this obstacle by directing the self-assembly of polystyrene–

poly(methyl methacrylate) — or PS–PMMA, a 'diblock copolymer' — on a lithographically patterned surface.

Diblock copolymers comprise two chemically distinct but individually homogeneous polymer chains joined end to end (Fig. 1). In bulk, repulsions between the unlike blocks induce the blocks to form 'microdomains', whose dimensions are set by the polymer's chain length. When the two blocks have roughly equal volumes, lamellar microdomains form, resembling a stack of playing cards, with each layer only a few tens of nanometres thick. In the absence of any guiding template or field, these lamellar stacks are randomly oriented.

But when diblock copolymers are deposited as thin films on substrates, chemical

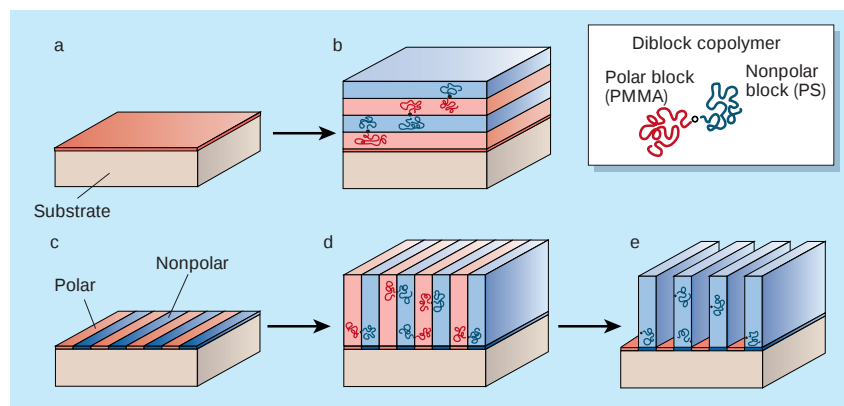


Figure 1 Self-assembly of block copolymers on substrates. Polystyrene–poly(methyl methacrylate), or PS–PMMA, is a diblock copolymer — it comprises two polymer chains, of PS (blue) and PMMA (red). The polar PMMA block adsorbs to a uniformly polar substrate surface (a), driving the PS and PMMA lamellae, with a period of about 50 nm, to lie parallel to the substrate (b). If, instead, the substrate is patterned with alternating polar and nonpolar stripes (c), with a period that is similar to that of the PS–PMMA, the block copolymer self-assembles epitaxially, with lamellae forming perpendicular to the surface and in precise register with the underlying pattern (d). Further chemical modification of the block-copolymer film, such as depolymerization of the PMMA block (e), can translate the original chemical pattern on the substrate into a template for patterned functional materials.

differences between the blocks can generate a preferential orientation; for example, polar blocks will wet polar substrates, but nonpolar blocks will wet the film's air surface. So, when polar substrates are coated with a film of PS-PMMA several lamellar spacings thick, adsorption of the polar PMMA block to the substrate and the nonpolar PS block to the air surface causes the lamellae to stack up with remarkable regularity² (Fig. 1b). Kim *et al.*¹ exploit differences in block polarity to generate 'standing' lamellae (Fig. 1d), by first creating a pattern of polar and nonpolar stripes on the substrate (Fig. 1c) with a repeat distance, or period, that closely matches the natural period of the block copolymer (which is 48 nm for the PS-PMMA they used). This reproduction of the substrate structure in the deposited overlayer is known as 'epitaxy'. The pattern, 50 × 400 μm overall, is created by extreme-ultraviolet interference lithography (EUV-IL) on substrates coated with a nanometre-thick, organic self-assembled monolayer (SAM); regions of the normally nonpolar SAM exposed by EUV-IL are then oxidized to generate the chemical pattern. When the periods of the pattern and copolymer are the same, the substrate pattern directs the block copolymer to assemble its lamellae vertically, in register with the pattern.

If the PMMA block is then depolymerized, the standing polystyrene lamellae that remain can be used as a template (Fig. 1e), for example for the deposition of magnetic metals³, or for patterned etching of the substrate⁴. Even more interesting would be applications in which the diblock itself — looking beyond PS-PMMA — has useful optical, electronic or magnetic properties. Alternatively, an active device could be created through selective chemical transformation: for example, cleavage of a polymethacrylate block to poly(methacrylic acid) would produce nanofluidic channels whose permeability could be controlled through pH or ionic strength⁵. The substrate patterns need not be limited to stripes; a second exposure to EUV-IL of the same substrate, rotated by 60°, would generate a pseudohexagonal lattice of lozenges, onto which arrays of cylinders could assemble vertically.

The epitaxial self-assembly described by Kim *et al.*¹ complements other work in which oriented block-copolymer films are used to generate, rather than replicate, the substrate pattern. For example, block-copolymer microdomains can be aligned with in-plane electric fields⁶, or through patterning the substrate on a length scale coarser than the microdomain spacing^{7,8}. Epitaxial self-assembly has the distinct advantage that precise registration of the microdomains with substrate features can be achieved: undulations of the lamellae⁹ are completely suppressed, yielding straight and narrow stripes. Kim *et al.* have fabricated stripes that are all 24 nm wide, running the full 400-μm



Figure 2 Precise patterning. Kim *et al.*¹ have used a striped polar–nonpolar substrate to control the self-assembly of the diblock copolymer PS-PMMA. In this scanning-electron-microscope image (corresponding to a top view of Fig. 1d), the light and dark regions are 'standing' lamellae of PS and depolymerized PMMA, respectively. On the right are straight, narrow stripes, each 24 nm wide, following the stripe pattern of the substrate; the stripes extend to lengths of up to 400 μm across the width of the substrate. On the left, where the diblock copolymer rests upon an area of unpatterned substrate, the PS and PMMA lamellae still stand edge-on, but their orientation in the plane of the substrate is random.

length of the substrate stripe in the best cases (Fig. 2). Moreover, they have produced regions up to 5 × 8 μm that are completely free of defects, even dislocations⁹ (which are lamellae that end prematurely, like a torn card inserted in the middle of the deck).

Dislocations destroy the translational order of the lamellar structure. They form easily (their energetic cost is small) and are difficult to eliminate. Consequently, many approaches^{6–8} to guided self-assembly can produce long-range orientational order, but they achieve only finite translational order. But by patterning the substrate, Kim *et al.* raise the energetic cost for dislocations and undulations — both of which move the lamellae out of register with the substrate pattern — so these detrimental features are less likely to form. The efficacy of epitaxy rests critically upon the match between the

period of the substrate pattern and the period of the diblock copolymer; when the former exceeds the latter by 5% or more, the lamellae tilt and twist to compensate, creating a defective pattern^{1,10}. But this commensurability requirement can be turned to an advantage, as decoration of the substrate with diblock immediately reveals defective regions in the underlying chemical pattern (such as the unpatterned region of substrate on the left in Fig. 2).

Kim and colleagues' method for substrate pre patterning requires specialized EUV tools and masks, but alternative methods for generating SAM-patterned substrates may be emerging. Microcontact printing of SAMs has typically been used to define features as small as 1 μm (ref. 11), but recent advances in contact printing¹² have demonstrated edge resolutions of 10 nm. If pushed to even finer resolution, contact printing could be used to pattern substrates for block-copolymer epitaxial self-assembly, using stamps that are replicas of complex masters written by electron-beam lithography. Such direct stamping would permit the fabrication of nonrepetitive patterns that could guide lamellae to curve, join, split or end, perhaps forming templates for electronic or nanofluidic circuitry. ■

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Developmental biology

Boundary lines

Seth S. Blair

Embryonic development is a complicated business, as a recent meeting bore witness. The signals generated at the boundaries between compartments were a key topic of discussion.

During development, multicellular organisms generate a complex and precisely patterned set of tissues from much simpler precursors. The mechanisms that accomplish this feat are diverse and, in many cases, still unknown. One of the best understood and most elegant

solutions is found in the fruitfly *Drosophila melanogaster*^{1,2}. Here, the cells that will eventually line the body surfaces are initially divided into a few large, spatially distinct 'compartments'. Specialized cells are then generated at the boundaries between compartments and act as organizers of further

development, producing long-range signals that subdivide adjacent tissues into new domains and new signalling centres. In the 30 years since *Drosophila* compartments were discovered by Antonio Garcia-Bellido and co-workers, similar mechanisms have been found in a variety of contexts, including vertebrate development. Boundaries and compartments were the unifying themes of a meeting held last month*.

Compartments in *Drosophila* (see, for example, Fig. 1) were first identified by the restrictions they place on cell lineage: although proliferating cells may intermix within a compartment, they never mix with adjacent cells in another compartment. This spatial segregation provides a simple mechanism for determining the location of compartmental boundaries. To what extent is this mimicked in other organisms? Similar lineage restrictions have already been found in vertebrates, for instance between dorsal and ventral cells in developing limbs^{3,4}, and between adjacent segments (rhombomeres) in the hindbrain⁵. Recent work has identified several more lineage restrictions in the developing brain⁵, including one between the midbrain and hindbrain (M. Brand, Max Planck Inst. Mol. Cell Biol. Genet., Dresden; A. Joyner, Skirball Inst., New York) (although see ref. 6), the midbrain–forebrain boundary, and two subdivisions of the forebrain — the zona limitans intrathalamica in the diencephalon (C. Kiecker, King's College London) and the boundary between cortex and striatum in the telencephalon (N. Osumi, Tohoku Univ. Grad. School Med., Sendai).

Intriguingly, in most cases where a lineage restriction has been found, specialized cells are formed at the boundary. In *Drosophila*, cells near the boundaries are specified by a short-range signal produced by one compartment that can be received only by cells in the adjacent compartment. In some cases the mechanisms that induce boundaries in flies and vertebrates seem to be quite similar. For instance, cells at the boundary between dorsal and ventral compartments of the developing fruitfly wing are specified by activation of the receptor protein Notch. This requires that the enzyme Fringe, which modifies Notch's sensitivity to its activators, be expressed only in the dorsal compartment^{1,2}. In vertebrates, the patterned expression of Fringe is required to specify cells near the boundaries between adjacent rhombomeres (D. Wilkinson, NIMR, London), and for the formation of the zona limitans intrathalamica (C. Kiecker).

In *Drosophila*, boundary cells then produce long-range signals; non-boundary cells judge their proximity to the boundary (and thus their position) through the amount of signal that reaches them. Are vertebrate

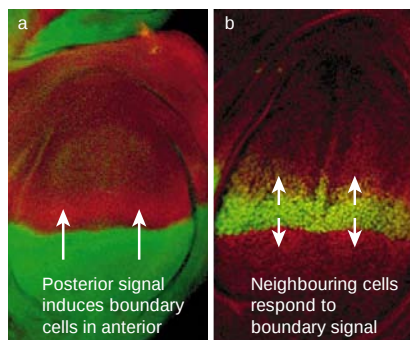


Figure 1 Compartments and boundaries in the developing wing of *Drosophila*. **a**, Hedgehog protein produced by the posterior compartment (green) induces adjacent cells of the anterior compartment to become boundary cells (the stronger red staining indicates the presence of the full-length form of the protein *Cubitus interruptus*, stabilized in response to Hedgehog). **b**, Decapentaplegic protein produced by boundary cells (green) induces different levels of response in cells near to or distant from the boundary (indicated by red staining, which represents the level of phosphorylated Mad protein).

boundary cells also the source of further signalling? In many cases the answer is yes, although the signals differ from system to system. Some are already well known: the midbrain–hindbrain boundary and the apical ectodermal ridge at the dorsal–ventral boundary of vertebrate limbs produce fibroblast growth factors and other signals that organize growth and patterning^{7,8} (A. Joyner). The zona limitans intrathalamica produces the signalling molecule Sonic hedgehog, and this is required for proper gene expression in adjacent forebrain regions (C. Kiecker).

What prevents cells in different compartments from intermixing? In some vertebrate examples, adjacent compartments express and require different adhesion or recognition molecules, which determine a cell's affinity for its neighbours and thereby influence its location⁵. For instance, different rhombomeres express different recognition molecules of the Ephrin and Eph receptor families, and signalling between these molecules inhibits cell migration between rhombomeres. In the forebrain, the separation of cortex and striatum depends on the compartment-specific expression of different cadherin cell-adhesion proteins (N. Osumi).

But there is also evidence that some key differences in cell adhesion or affinity might be limited to cells at compartment boundaries, rather than distributed throughout each compartment. In *Drosophila*, the signalling that produces boundary cells is also required to prevent cells in different compartments from intermixing^{1,2}. In vertebrates, evidence for boundary-specific cell affinities includes the fact that cells at rhom-

bomere boundaries are probably induced by high levels of Notch signalling, and activating Notch in non-boundary rhombomere cells causes them to preferentially migrate into the boundary region (D. Wilkinson). Moreover, ventrally migrating neurons stop at the cortex–striatum boundary, hinting that the boundary cells have different adhesive properties (N. Osumi).

In *Drosophila*, less is known about the recognition or adhesion molecules that might keep cells in adjacent compartments from intermixing; only one candidate molecule has been identified, and it is not essential². Screens for new regionally expressed genes continue (T. Kornberg, Univ. California San Francisco; H. McNeill, Cancer Research UK, London; J.-P. Vincent, NIMR, London), and these may yet uncover boundary-specific or compartment-specific recognition molecules. But an alternative hypothesis was also discussed: that the signalling between compartments not only induces boundary cells, but also gives them a polarity. So, the signal produced by one compartment might directly repel the cells in the adjacent compartment. For instance, directional Notch signalling across the dorsal–ventral boundary in the fly wing may modify the actin cytoskeleton on the side of the cells that is nearest the compartment boundary — potentially changing their direction of migration (K. Irvine, Rutgers Univ., Piscataway).

For all the 'true' compartments mentioned above, there are many more counter-examples, where precisely defined regions of gene expression and tissue formation are not specified by cell lineage. Cells can migrate in and out of these regions and change their fate; examples discussed include regions of the fruitfly thorax (G. Morata, Univ. Autònoma Madrid; J. Modolell, Univ. Autònoma Madrid), and the Sonic-hedgehog-expressing region of vertebrate limbs (C. Tabin, Harvard Medical School, Boston). Even the cells at the boundaries of forming vertebrate somites — the segmented blocks of tissue that will produce portions of the muscles, skeleton and skin — are surprisingly changeable (P. Kulesa, Stowers Inst. Med. Research, Kansas City).

In the end, the difference between these more flexible regions and lineage-defined compartments may simply be one of control. *Drosophila* lineage compartments are maintained by the stable inheritance of a given state of expression of specific 'selector' genes. The more flexible regions may be maintained instead by signalling between cells; cells that wander outside the region of optimal signalling change their identities. Can these regions also produce boundary cells? There is no reason as yet to suppose otherwise: such cells could be induced at the junction between any two cellular groups, however those regions are maintained.

*Boundaries in Development: 30 Years of Progress. EMBL, Heidelberg, Germany, 14–17 June 2003.

There are other unanswered questions. The mechanisms underlying signalling between cells were hotly debated: is signalling mediated by diffusion, or active transport, or by cells extending finger-like filopodia (S. Eaton, Max Planck Inst. Mol. Cell Biol. Genet., Dresden; T. Kornberg)? And, as several researchers pointed out, it is not known whether the long-range signals generated by boundaries can explain several important processes, such as growth control and regeneration (A. Garcia-Bellido, Univ. Autonoma Madrid; L. Wolpert, University College London). The concept of long-range signalling is at odds with older models, in which every cell was thought to have a molecular address that could be recognized by its

neighbours. Developing cells are exceedingly clever, and it is possible that they are still hiding Something Big.

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Neurobiology

A new way to network

Patrick Mehlen

Guidance molecules called semaphorins ensure that nerve cells grow in the correct direction during development. It now appears that one semaphorin interacts with integrin proteins to produce another effect.

During the development of the nervous system, nerve cells must extend projections called axons over considerable distances to construct a complex network of connections. How axons extend in the correct direction is conceptually simple: the extremity of the axon — the growth cone — explores its local environment for molecular cues, which guide it along the right trajectory. In practice, however, guidance of the growth cone is a complex, tightly regulated mechanism that involves several different families of guidance molecules. Some of these cues are soluble and can act at a considerable distance; others are bound to the cell membrane and act locally. Some cues attract the growth cone, others repel it and still others can act as both attractants and repellents¹. One group of guidance molecules is the aptly named semaphorin family². On page 398 of this issue, Pasterkamp *et al.*³ reveal a new way in which one type of semaphorin acts on nerve-cell axons to ensure that the brain develops normally.

More than 20 semaphorins have been identified, but only a few have been shown to be involved in the development of the nervous system². Others have been implicated in immune-cell function. It now appears that one member of the family — *Sema7A* — has a dual role, and functions during brain development as well as modulating the activity of immune cells. Pasterkamp and colleagues³ observed that *Sema7A* is expressed during neural development in mice, and that the brains of mice genetically engineered to lack this protein fail to develop normally. The

authors focused on the part of the brain that determines the sense of smell — the central olfactory system — and showed that *Sema7A* is normally present along the trajectory of a bundle of axons called the lateral olfactory tract. Strikingly, in the mice lacking *Sema7A*, this axon bundle had grown in the correct direction, but it was substantially smaller than normal. Semaphorins normally function as growth-cone repellents, but these results indicate that *Sema7A* functions in the olfactory system as neither a repellent nor an attractant. Instead, it seems to encourage axon outgrowth, in a direction that is probably dictated by other cues.

But how does *Sema7A* promote axon outgrowth? Other semaphorins are thought to guide developing axons by interacting with at least two families of receptors present on the growth-cone membrane: the neuropilins and the plexins². The classical view is that binding of semaphorins to either type of receptor sets off a chain of intracellular signalling events within the growth cone, which in turn modulates its pathfinding (Fig. 1, overleaf). Could *Sema7A* be exerting its growth-promoting effects in the same way? Previous *in vitro* protein-binding studies had suggested that *Sema7A* interacts with PlexinC1; furthermore, both PlexinC1 and *Sema7A* are often present within the same regions of the brain⁴. To investigate further, Pasterkamp *et al.* engineered mice lacking functional PlexinC1. But these mutant mice did not show the same brain abnormalities as the *Sema7A*-mutant animals, suggesting that PlexinC1 is not, after all, used by *Sema7A* to promote axon outgrowth.



100 YEARS AGO

It is known that salt (NaCl) at a temperature of 200°C is phosphorescent; during a course of experiments in June last I found that radium bromide induces phosphorescence at ordinary temperatures. The following is a convenient way of observing the phenomenon. Fill a wooden match-box with table salt removed from the inner portion of a block; press the radium bromide tube into the yielding mass and just barely cover it with the substance. If it be now put on one side for a few hours, say into one of the compartments of a chest of drawers, on opening the box in the dark all round the tube will be found to phosphoresce with a white light, but, unlike zinc blende and barium platinocyanide, the salt continues visibly to phosphoresce after removal of the radium bromide... The image of the visible portion round and where the radium bromide tube has lain is impressed on a photographic plate in thirty minutes, but only very faintly in two or three minutes.

From *Nature* 23 July 1903.

50 YEARS AGO

Evidence for 2-chain helix in crystalline structure of sodium deoxyribonucleate. Watson and Crick [*Nature* 171, 737, 1953] have proposed a structure for sodium deoxyribonucleate consisting of two co-axial helical chains related by a diad axis. We have shown [page 740 of the same issue] that the main features of their structure are consistent with certain important features of our X-ray diagrams of structure *B* (the high-humidity less-ordered form of the salt). A subsequent closer investigation of density and water content in relation to the prominent equatorial spacing, and also of equatorial intensities calculated from a projection of the proposed structure (kindly provided by Watson and Crick), makes it clear that in detail the structure is not consistent with the observed equatorial reflexions. Both density and intensity considerations lead us to favour a more compact helical structure in which the phosphorus atoms lie on a helix of radius about 8.5 Å. rather than 10 Å. This value also lies within the range of spread of the more diffuse layer-line peaks. We are more concerned here, however, with evidence which confirms in principle the type of structure suggested by Watson and Crick, than with criticism on points of detail.

Rosalind E. Franklin and R. G. Gosling
From *Nature* 25 July 1953.

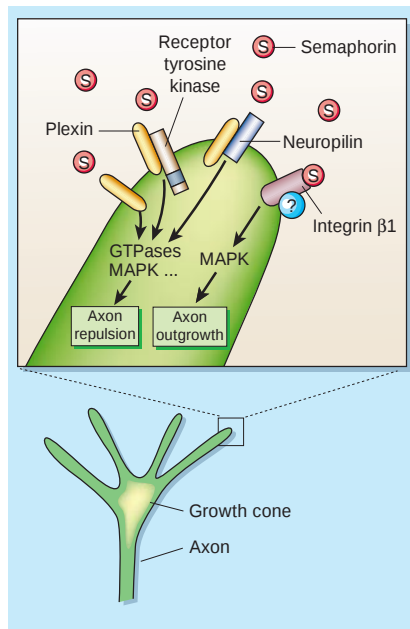


Figure 1 Effects of semaphorins on the development of the nervous system. Several receptor complexes have been proposed to interact with semaphorins present in the environment of developing nerve cells. For instance, semaphorins can interact with a plexin receptor, a complex formed by a plexin and a receptor tyrosine kinase, or a complex formed by a plexin and a neuropilin to steer the growing axon (see ref. 2 for a review). Pasterkamp *et al.*³ have now found that one semaphorin, Sema7A, interacts with integrins, thereby stimulating the growth of the axon. The semaphorin–receptor interactions produce these effects through cascades of intracellular signals that include small GTPases or mitogen-activated protein kinases (MAPKs).

In search of alternative receptors, the authors scanned the amino-acid sequences of different semaphorin proteins for clues. They observed that several semaphorins, including Sema7A, contain the arginine–glycine–aspartate (RGD) sequence, which is frequently associated with binding to integrin proteins. Integrins are a large family of membrane-spanning receptors that are involved in cell adhesion⁵. They usually function as dimers of an α -subunit and a β -subunit, of which there are many different types. Using a panel of inhibitors to block specific integrin subunits, together with mutant Sema7A proteins lacking the RGD sequence, Pasterkamp *et al.* determined that a β 1-type integrin subunit is required for the growth-promoting activity of Sema7A (Fig. 1). Moreover, addition of Sema7A to cultured neurons led to intracellular signalling events that included the activation of the key enzymes focal adhesion kinase and mitogen-activated protein kinases — enzymes known to be activated by integrins and to be required during axonal guidance^{6,7}.

The proposal that integrins are receptors for semaphorins opens up a new vista on their role during brain development. Integrins have already been implicated in axon guidance, but until now it was thought that they simply allowed nerve cells to attach firmly within an environment that had already been deemed ‘permissive’ by other guidance signals⁸. Pasterkamp *et al.* have shown, however, that integrins have a more direct part to play. Specifically, they are receptors, or part of a receptor complex, for a cue that stimulates axonal outgrowth.

As usual, this discovery prompts yet more questions. Other semaphorins, and proteins belonging to other families of guidance cues, also contain RGD motifs. Do they also interact with integrins? As integrins usually function as dimers, what is the nature of the other half of the integrin receptor used in Sema7A signalling? Is it an α -integrin, or does an alternative receptor substitute for the α -subunit? Is the growth-cone-repelling activity of semaphorins also transmitted through integrins? Finally, Sema7A is also a potent modulator of immune responses. Does this activity require interaction with integrins?

Elsewhere in this issue, Serini *et al.*⁹ propose that Sema3A proteins, although they do

not have an RGD motif, control blood-vessel development by altering integrin activity. So a deeper understanding of the intracellular signalling pathways that are activated by semaphorins through integrins will provide insight into a variety of physiological processes, from vascular and neural development to immunity. The semaphorin–integrin interaction may also have implications for disease, shedding light on events such as the formation of new blood vessels in cancers, harmful immune responses, and how nerves regenerate after injury. ■

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Astronomy

An early stellar nursery

Philip Solomon

Searching for distant objects in our Universe is equivalent to looking back in time, to the early origins of stars and galaxies. The most distant object known shows the earliest evidence of star formation.

In the astronomical hunt for ever more distant objects, and hence a window on the early Universe, the current leader¹ is J1148 + 5251 at a redshift of 6.4. The light we now see from this particularly luminous object was emitted only 800 million years after the Big Bang, making it the youngest object known. J1148 + 5251 is a quasar (from ‘quasi-stellar’ object). Whereas stars are powered by nuclear fusion, a quasar’s power is derived from gravitation: quasars are powered by matter from rotating accretion disks spiralling into massive black holes at the centres of galaxies.

Observations of an object as young as J1148 + 5251 could reveal much about the evolution of galaxies early in the history of the Universe, and about the relation between the formation of stars and massive black holes. New data are now reported, by Walter *et al.*² on page 406 of this issue, and by Bertoldi *et al.*³ in a complementary article in *Astronomy and Astrophysics*. These authors have detected radiation at millimetre wavelengths from molecules of carbon monoxide, indicating the presence of a large mass of interstellar

molecular gas, and from which the presence of molecular hydrogen (the dominant component in molecular clouds) can be inferred. This is the raw material from which stars form. These measurements, combined with the observation of strong emission at far-infrared wavelengths from interstellar dust⁴ in this very young galaxy, point to an ongoing burst of star formation that began only a short time after the Big Bang.

The combination of high luminosity at far-infrared wavelengths and a large mass of molecular gas and dust is an accepted signature of star formation in galaxies. Young stars embedded in the molecular clouds heat the interstellar dust, which then radiates at infrared wavelengths. In the local Universe, many spiral galaxies show significant infrared luminosity from star formation, and the most powerful galaxies — ultra-luminous infrared galaxies⁵ — all have large masses of molecular gas⁶ and CO emission similar to that seen in the distant J1148 + 5251. Most ultraluminous galaxies seem to have formed from collisions between separate galaxies⁵, and their molecular gas is

found concentrated in rotating disks or rings a few thousand light years across⁷ — a central region much smaller than the galaxies but much larger than a quasar accretion disk. An analysis of the CO emission from a quasar at lower redshift than J1148 + 5251 showed that the molecular gas is also distributed in a star-forming ring with a size of about 6,000 light years⁸.

From the observed infrared luminosity of J1148 + 5251, Walter *et al.*² and Bertoldi *et al.*³ have calculated the rate of star formation in its galaxy. It is equivalent to the creation of about 3,000 Sun-like stars each year — a thousand times greater than the star formation rate in the entire Milky Way, and a few times greater even than that of ultraluminous galaxies. Star formation on this scale is a major event in the evolution of a galaxy.

It is possible that some of the far-infrared radiation originates from dust heated by the quasar itself. However, there is another very strong line of evidence in favour of a major episode of star formation in this galaxy. The very existence of a large mass of interstellar molecules and dust is proof that substantial star formation is taking place, or occurred at an even earlier time. In addition to carbon monoxide gas, the small dust particles are composed of what astronomers refer to as heavy elements or 'metals', such as carbon, oxygen, silicon and magnesium. All of these elements were produced after the Big Bang, inside stars, by a process known as nucleosynthesis⁹, and then ejected into the interstellar medium. This interstellar matter provides the raw material for a new generation of stars and planets. There are already indications from the spectrum of the optical-wavelength emission¹ that high-redshift quasars show strong emission from metals, but this does not by itself indicate the amount or extent of star formation. The molecular gas and dust around J1148 + 5251 clearly show that the process of chemical enrichment was well advanced only 800 million years after the Big Bang. For this to occur in such a short time implies that the enrichment is due to the formation and evolution of high-mass stars, the same type of star responsible for the heating of the dust.

J1148 + 5251 is not the first high-redshift quasar found to have strong far-infrared radiation from warm dust. More than 20 quasars at redshift, z , higher than 3.8 have been identified¹⁰ whose high far-infrared luminosity is associated with star formation. CO emission has been detected in more than 13 quasar host galaxies with z larger than 2, including the previous high-redshift record-holder¹¹ at $z = 4.7$. In addition, a large number of far-infrared sources have been found from 'blank field' surveys^{12,13}, most of which are also very luminous and have redshifts in the range 1 to 3.5 (ref. 14). For only two of these have CO spectral lines been reported¹⁵, but it is likely that CO emission will be found



Figure 1 East meets west. The Very Large Array (left), on the plains of New Mexico, USA, is a Y-shaped configuration of 27 radio telescopes. In the Hautes Alpes of France, an array of five (now six) antennas forms the Plateau de Bure Interferometer (right). Arrays of telescopes such as these, operating synchronously, are equivalent to a single antenna dish up to many kilometres in diameter, and hence offer more power for resolving distant objects. Walter *et al.*² and Bertoldi *et al.*³ have used data from both arrays in their study of the most distant astronomical object known, a quasar called J1148 + 5251, and have uncovered evidence for star formation at an early stage in the history of the Universe.

in most of them now that their redshifts are known. These dust-enshrouded molecular-gas-rich galaxies account for about half of all star formation in the Universe.

Among all of these, the galaxy hosting J1148 + 5251 is the most distant and the earliest example of a star-forming region. That also makes it the only example so far of star formation occurring at a time before the hydrogen between galaxies (the intergalactic medium) was completely ionized by ultraviolet radiation from other galaxies and quasars, a time in the evolution of the Universe known as the epoch of reionization. The presence of even a few per cent of un-ionized intergalactic hydrogen atoms is a clear indicator that the Universe was in the early stages of lighting up, following the cooling off of the intergalactic medium a few hundred thousand years after the Big Bang.

The ability to observe molecular emission from such distant objects is due to the remarkable sensitivity of the instruments used by Walter *et al.*² and Bertoldi *et al.*³ — the National Radio Astronomy Observatory's Very Large Array and the Plateau de Bure Interferometer of the Institut de Radio Astronomie Millimétrique (Fig. 1). In some cases there is also help from magnification of the signal by gravitational lensing along the line of sight, although this is probably a small effect¹ in J1148 + 5251. In addition, there is what astronomers refer to as a 'negative K correction' for the highly redshifted radiation from thermally excited rotational energy levels¹⁶ of molecules. For a given telescope operating at an approximately fixed frequency, it is possible to observe CO emission from redshifted galaxies simply by observing a spectral line from a higher rotational energy level and higher rest frequency, instead of lowering the frequency to match the redshift.

This switching of spectral lines means that it is no harder to detect CO when the source is at a redshift of 6 than when it is at a redshift of 1 (provided that the source redshift is well known and the gas is not extremely cold).

Nonetheless, these measurements are right at the limit of existing telescopes. The constraints will be lifted by the new generation of instruments, particularly the Atacama Large Millimeter Array of telescopes being built by an international collaboration in Chile. With increased resolution, sensitivity and bandwidth, this array will produce images of interstellar molecules and dust with the clarity that the Hubble Space Telescope has brought to optical astronomy. A fuller understanding of the formation of galaxies and stars in the early Universe awaits us. ■

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Obituary

Ira Herskowitz (1946–2003)

Ira Herskowitz died on 28 April 2003 of pancreatic cancer. He was a scientist of style and influence. Genetics was his path to truth, and he used that approach to make a string of important discoveries in cell and molecular biology.

Herskowitz made an impression early on, as a graduate student with Ethan Signer at the Massachusetts Institute of Technology (MIT) in Cambridge, Massachusetts. He took on the problem of how a protein can activate — increase the transcription of — a gene. Transcriptional activators (as opposed to repressors) were not much in vogue in the late 1960s, and there were no good ideas as to how they might work. Herskowitz studied a protein called Q, which is encoded by the bacterium-infecting virus (bacteriophage) λ and is required for the transcription of a group of other λ genes. He showed that a single DNA site in the λ genome is required for Q to activate all of these target genes. The result revealed that the so-called λ late genes must be transcribed into a single, long messenger RNA, and it set the stage for a full understanding of the molecular workings of Q that is only now coming to fruition.

Thus began a lifelong love affair with bacteriophage λ . Although his lab formally stopped working on the virus in the 1980s, Herskowitz remained engrossed by the subject. In the years before his death he spoke of writing a small book to tell the story of the λ *cII* and *cIII* gene products. Synthesized when λ first enters a host cell, these proteins sense the health of the cell and decide whether the virus should replicate itself and destroy the cell, releasing its progeny in the process, or whether it should silence its genes and integrate into the host genome. The book would have expanded on his influential review on the subject written thirty years ago.

Herskowitz left MIT in 1972 for the University of Oregon, Eugene, where he initiated a series of classic studies on the single-celled yeast *Saccharomyces cerevisiae*. He realized early on (and was instrumental in teaching the rest of us) the power of using yeast as a model organism to study fundamental biological problems. Like humans, other mammals, plants and insects (but unlike bacteria), yeast is a eukaryote — an organism whose cells have defined nuclei. Not only that, but genetic analyses with yeast are about as easy to do as they are with bacteria. This was the opening that fired Herskowitz's



Scientist who brought yeast genetics to the forefront of molecular and cell biology

imagination, and it was here that he made his greatest impact.

Herskowitz's studies on yeast 'mating-type switching' had a celebrated outcome. *S. cerevisiae* has two mating types, or sexes, and the cells, as they divide, switch back and forth between them. A set of simple experiments confirmed a most unlikely-seeming scenario: programmed DNA rearrangement was responsible for the switching. Few steeped in the world of bacteriophage λ , where proteins bind to DNA to turn genes on and off, would have been prepared to make Herskowitz's leap to a world in which gene control was effected by gene rearrangement.

In 1981, Herskowitz moved to the University of California, San Francisco, where he remained for the rest of his career. There, he initiated a series of studies that revealed fundamental features of eukaryotic cell growth and division. For example, cells often divide asymmetrically, one progeny cell differing

in important ways from the other. Herskowitz showed how this occurred in yeast: a key molecular determinant (in this case, a particular mRNA) is distributed to only one of the two progeny cells. The protein produced by this mRNA then initiates a developmental programme that distinguishes one cell from the other. To take another example, every time a yeast cell divides, a molecular mark is left on its surface, thereby recording the history of its cell divisions. Herskowitz showed how the cell then uses these marks to tell which end is which and to establish its subsequent patterns of growth and division.

Herskowitz also explored other aspects of cell biology, including signal transduction, cell-cycle progression, polarized cell growth, chromatin structure, meiosis, gene expression and RNA localization. Virtually all of these studies exploited properties of the yeast mating machinery that he was instrumental in characterizing. Thus, from modest and seemingly specialized questions about the mating behaviour of a single-celled organism, Herskowitz created a body of work with implications for many different branches of molecular and cell biology.

Herskowitz's science was always simply described, yet rich in metaphor and analogy. He trained more than 70 graduate students and postdoctoral fellows. He also helped many more scientists to clarify their ideas, design their experiments and write their papers. A discussion with Ira could entice a biologist, even one who had never studied with him, to delve into new experimental realms. Ira's style of presentation — the simple story, the coloured chalk and the hand-drawn diagrams (where simple arrows inevitably stood in for detailed mechanisms) — became a standard to be emulated. A biological polyglot, he chose his yeast projects with an eye to solving problems under study elsewhere with more complex organisms. He was the master of the simple, telling experiment. He was a gentle man and a gentle scientist. He will remain an unforgettable figure.

Alexander Johnson and Mark Ptashne

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Optics

Laser light sees through clouds

Appl. Phys. Lett. **83**, 213–215 (2003)

Laser light can penetrate thick cloud, say François Courvoisier *et al.*, improving the prospects of using laser beams for telecommunications and remote sensing in the atmosphere. The researchers show that very intense laser pulses are able to resist scattering by cloud droplets.

Above a certain power threshold, laser pulses form localized waves, called light filaments, that maintain their integrity as they travel through space (rather like the solitons first observed in tidal-bore-like water waves). Courvoisier and colleagues have studied the transmission of light filaments through water droplets that are tens of micrometres in diameter, mimicking cloud droplets. The filaments, typically about 150 μm across, are surrounded by a bath of unfocused photons that act as an energy reservoir, replenishing and 'rebuilding' a filament when it loses energy and intensity by scattering. This would enable light filaments to pass through virtually 'opaque' clouds; indeed, the filaments even survive a passage through large (around 0.1 mm) droplets of black ink.

Philip Ball

Microbiology

Prion as passport

J. Exp. Med. **198**, 5–17 (2003)

The bacterium *Brucella abortus* poses quite a threat: it causes spontaneous abortion in livestock, and produces fever, arthritis and meningitis in humans. *B. abortus* infections are generally long-lasting, and it is thought that this is because, although the bacterium is engulfed by pathogen-hungry macrophage cells, it is not destroyed and instead lives there undetected.

Masahisa Watarai and colleagues now show that *B. abortus* gains safe entry into macrophages by hijacking the cellular prion protein (PrP^C). The process requires a secreted *B. abortus* protein called Hsp60, which recognizes PrP^C on the macrophage surface. The bacterium is then bundled into the cell, swaddled in a piece of macrophage membrane. Watarai *et al.* further show that mice lacking the gene for PrP^C are immune to *B. abortus* infection.

In an accompanying commentary, Adriano Aguzzi and Wolf-Dietrich Hardt suggest that the finding may point to a role for PrP^C. So far there is no confirmed function for this normal form of the prion protein, although the diseased form (PrP^{Sc}) causes brain-wasting diseases such as Creutzfeldt–Jakob disease in humans. Hsp60 is found in bacteria and all eukaryotic cells,

and is known to induce inflammation and immune responses. Perhaps PrP^C is part of a generalized threat-detection mechanism that senses Hsp60 — a mechanism that is cunningly abused by *B. abortus*. Tom Clarke

Photonics

Drawing a line in silicon

Adv. Mater. **15**, 1167–1172 (2003)

Photonic crystals — materials that are impermeable to light of selected wavelengths — seem ideal for making light-based microchips, but only if they can be patterned with defects that form light-carrying channels. Nicolas Tétéault *et al.* have devised a way of writing lines of about a micrometre wide into thin films of silicon photonic crystals using a laser beam.

The photonic crystal is a silicon film perforated with a regular array of spherical voids, made by casting the silicon around a colloidal crystal of stacked silica microspheres and then etching the silica away. The porous silicon, which is amorphous (non-crystalline) and contains hydrogen, is compatible with standard silicon-chip electronic circuitry and excludes infrared radiation at the 1.5- μm wavelength used for optical telecommunications.

To open up channels, or waveguides, for shunting light around on a 'photonic chip', the researchers shine a pencil beam of green laser light onto the holey film. Local heating melts the amorphous silicon, which forms nanocrystals as it cools. This nanocrystalline silicon has a different refractive index from the amorphous form, and acts as a transparent waveguide within the photonic-crystal matrix. Light confined in this way can be guided around tight bends, without the losses that would occur in conventional miniaturized waveguides in which confinement relies simply on reflection at the walls.

Philip Ball

Hearing

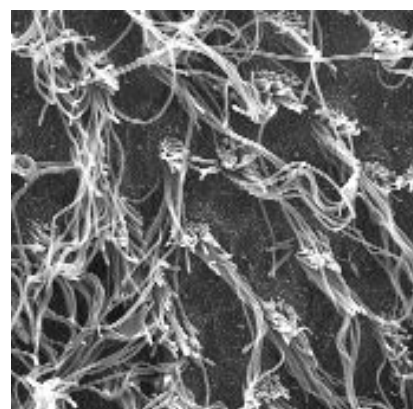
Spin the chicken

J. Neurosci. **23**, 6111–6122 (2003)

People treated with large doses of certain antibiotics, such as streptomycin, can suffer permanent hearing and balance problems as a side effect. This is because caspase genes are activated in sensory hair cells (pictured) in the inner ear, causing the cells to die off. *In vitro* studies have found that simultaneous treatment with caspase inhibitors can reduce the damage.

Jonathan I. Matsui *et al.* now show that a similar strategy may work *in vivo*. The authors found that when chickens were treated with streptomycin, up to 60% of their hair cells died. But when the inner ear was infused with a broad-specificity caspase inhibitor, up to 95% of the hair cells survived.

Matsui *et al.* checked to see whether the



cells were still active by rotating the chickens and watching their eyes. Functional hair cells detect head motion as well as sound, so that when the head moves, animals can stabilize their gaze by making compensatory eye movements. Chickens treated with caspase inhibitors showed improved eye responses, indicating that their hair cells were working. Whether or not the drug has a similar effect in humans, however, remains to be seen.

Helen R. Pilcher

Plant physiology

Heavy metal and snails

New Phytol. **159**, 461–469 (2003)¹

New Phytol. **159**, 453–459 (2003)²

Many plant species extract heavy metals, such as zinc, nickel or selenium, from soil and concentrate them in their leaves. Such hyperaccumulation may help plants to grow on contaminated soils, but could it also protect against invertebrate herbivores? The answer appears to be yes for caterpillars, but no for snails.

Brady Hanson and colleagues¹ gave Indian mustard (*Brassica juncea*) seedlings selenium-rich water, producing leaves containing around 0.1% selenium. Caterpillars of the cabbage white butterfly (*Pieris rapae*) avoided such leaves and died of selenium poisoning if they were their only food source. But land snails (*Mesodon ferrissi*) showed no ill effects, and even actively sought leaves with a high selenium content.

Simone B. Huitson and Mark R. Macnair² used a different approach: they crossed a species of cress that hyperaccumulates zinc (*Arabidopsis halleri*) with the related *A. petraea*, to produce plants containing a range of zinc concentrations when grown in the same zinc-rich soil. Garden snails (*Helix aspersa*) did not discriminate between plants with high or low zinc levels, although they did tend to avoid pure-bred *A. halleri*.

These studies do not settle the role of hyperaccumulation in plant defence, but they do confirm what all gardeners know: the voracity of snails is almost impossible to deflect.

Christopher Surridge

Reversal of sex roles in nuptial feeding

Female Zeus bugs subvert the traditional notion that amorous gifts are a male preserve.

Males of many animals provide females with 'nuptial gifts', such as prey items or glandular secretions, during courtship and mating¹. Traditionally, nuptial feeding is viewed as a form of paternal investment in offspring^{2–4}. Although other explanations have been proposed^{5–7}, the focus remains gender-specific, with a male donor and female recipient. Here we show that females of the extraordinary insect *Phoreticovelia disparata* provide food for males during mating. This previously undescribed reversal of gender roles indicates that nuptial feeding might not be related to paternal investment.

The recently discovered genus *Phoreticovelia* (Heteroptera: Veliidae)^{8,9} consists of semi-aquatic insects that inhabit tropical rivers. They are gregarious and nocturnal predators; swarms scavenge the water surface at night for food. Female morphology and the mating habits of these Zeus bugs (so called because, according to legend, Zeus consumed his first wife, Metis) indicate that males might be feeding on female glandular secretions during mating (Fig. 1).

We tested this possibility by using *P. disparata* collected from the Upper Mulgrave River, Queensland, Australia. We first primed individual females kept with or without males by feeding them on radiolabelled (³H) or non-labelled *Drosophila melanogaster* for 10 days (*n* was 16, 17, 19 and 20, respectively). New focal males were then allowed to ride females for four days while they were offered only non-labelled food. All focal individuals were subsequently dissolved (in 100 µl 50 mM NaOH and 5 ml scintillation fluid) and the amount of radioactive label was measured in a scintillation analyser.

Focal males riding females fed with non-labelled food did not contain any label (males versus control vials; *t*-test, d.f. = 44, *P* = 0.819). However, focal males riding labelled females contained labelled material (mean d.p.m. (adjusted for background radiation) ± standard error; 11.14 ± 1.48 versus -0.58 ± 0.46; ANOVA, *F*_{1,68} = 65.03, *P* < 0.001), irrespective of the presence or absence of a male during priming (*F*_{1,68} = 0.51, *P* = 0.474). This confirms that material passed from the female to the male, at a rate consistent with the male consuming a few per cent of his body weight of female gland secretion per day.

Females produce glandular secretions only when ridden by a male. We removed all glandular secretions from females (Fig. 1a), isolated them individually with or without a male (*n* = 10 for both) for 13 days, and then

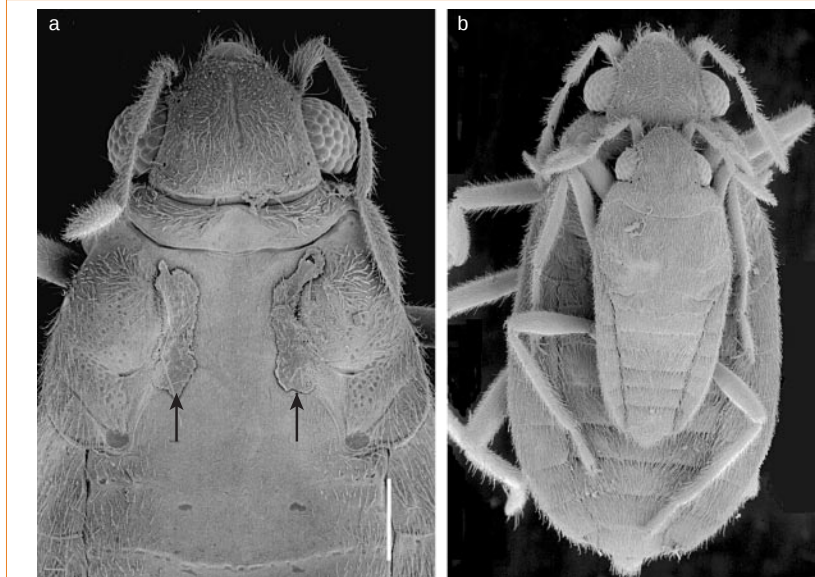


Figure 1 Male Zeus bugs feed on females during mating. **a**, Females are equipped with a unique pair of dorsal glands, producing a secretion that upon drying creates two oblong, whitish patches (arrows). Scale bar, 0.2 mm. **b**, Males lack these glands, are much smaller than females (*P. disparata*; 1.2 as opposed to 2.0 mm), and ride adult females, as well as late-instar female nymphs, almost permanently without genital contact (mean mating duration in large laboratory populations; 8.2 ± 2.0 days, *n* = 10). During riding, the male mouthparts are located at the opening of the female dorsal glands. Adult sex ratio in the field is slightly male biased (1.2:1) and more than 95% of adult females carry a male on their back.

scored the distribution of glandular secretion. The former, but not the latter, females regenerated their patches (Mann–Whitney *U*-test; *P* = 0.002). Further, the lifespan of starved males increased by about 50% when they were kept with a female compared with those kept alone (2.4 ± 0.2 versus 1.6 ± 0.2 days; *t*-test, *P* = 0.014), but there was no significant difference in survival when males were fed (10.6 ± 1.4 versus 13.1 ± 1.6 days; *t*-test, *P* = 0.252; ANOVA interaction, *F*_{1,79} = 3.99, *P* = 0.049), suggesting that males benefit from feeding on females.

We also monitored the lifetime reproductive performance of individual females (fed with one cricket nymph per day) kept with or without a male (each *n* = 15). The presence of a male did not significantly affect female lifespan (15.5 ± 1.9 versus 14.3 ± 2.4 days; *t*-test, *P* = 0.699) or lifetime fecundity (3.9 ± 1.0 versus 4.5 ± 0.8 eggs; *t*-test, *P* = 0.688). The hatching success of eggs did not differ between treatments during the first (75% versus 69%; $\chi^2_1 = 0.27$, *P*_{adj} = 0.303) or second (75% versus 75%; $\chi^2_1 = 0.12$, *P*_{adj} = 0.365) week of the experiment, but was higher among females kept with a male during the third week (100% versus 33%; $\chi^2_1 = 5.14$, *P*_{adj} = 0.041). This suggests that females do not need to mate more than every two or three weeks to maintain full fecundity and fertility.

We have demonstrated a novel form of nuptial feeding, the evolution of which is unrelated to paternal investment. In conventional gift-giving species, males gain reproductive success by providing nuptial gifts¹. In Zeus bugs, females might benefit from feeding and carrying males. Our results indicate that female gift-giving is not necessary to ensure a regular sperm supply¹⁰, and that instead it might have evolved to reduce costs imposed by males, in the form of cannibalism, kleptoparasitism and other forms of interference.

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Psychophysics

Bees trade off foraging speed for accuracy

Bees have an impressive cognitive capacity^{1–4}, but the strategies used by individuals in solving foraging tasks have been largely unexplored. Here we test bumblebees (*Bombus terrestris*) in a colour-discrimination task on a virtual flower meadow and find that some bees consistently make rapid choices but with low precision, whereas other bees are slower but highly accurate. Moreover, each bee will sacrifice speed in favour of accuracy when errors are penalized instead of just being unrewarded. To our knowledge, bees are the first example of an insect to show between-individual and within-individual speed–accuracy trade-offs.

Psychophysicists studying stimulus discrimination in animals have been mainly concerned with the accuracy of discrimination, not with its speed. But in humans (see, for example, ref. 5) there is a tight relationship between the two. We therefore investigated how bumblebees might achieve a compromise between speed and accuracy while foraging from a ‘virtual’ meadow.

A nest box was connected to a flight arena (100 cm × 70 cm × 70 cm); one of the walls (70 cm × 70 cm) was a translucent Plexiglas screen. Virtual ‘flowers’ (coloured circles of diameter 25 mm) were projected onto the screen by a data projector controlled by a PC and Java software. The screen contained 46 holes, each 5 mm in diameter, arranged in a hexagonal pattern; the distance between neighbouring holes was 10 cm. Sucrose and other solutions could be applied from behind the screen with a micropipette.

Virtual flowers were projected onto 8 of the 46 possible locations on the screen in such a way that one hole in the screen formed the centre of each flower (Fig. 1). Four of the virtual flowers (‘targets’) were rewarding with 10 µl sucrose solution (2 M). These were coloured blue; the colour was adjusted to $R=0$, $G=0$, $B=255$ in the eight-bit RGB (red–green–blue) colour model. Four other similarly coloured virtual flowers acted as ‘distractors’ (unrewarding virtual flowers: $R=0$, $G=70$, $B=255$). Distractor flowers were charged with a droplet of water.

Flower locations were randomized every hour during training, and between individual foraging bouts during tests. After two days of training, bees were tested individually for three consecutive foraging bouts. Choice time was assessed as flight time between flowers; a decision was recorded when a bee made contact with the landing platform.

The average percentage of correct choices was $62 \pm 11.2\%$ (mean $\pm$ s.d.), and bees, as a group, behaved significantly differently from

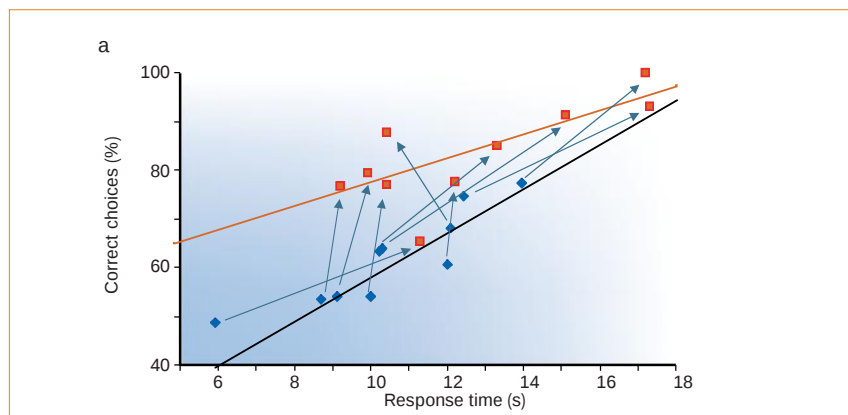
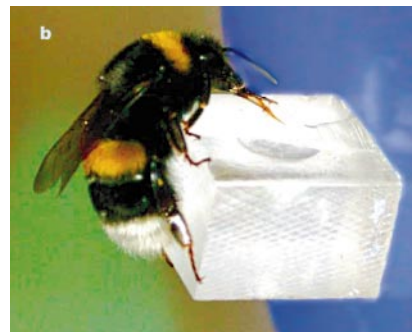


Figure 1 Bumblebees can choose wisely or rapidly, but not both at once. **a**, Interindividual correlation between response time and accuracy of bees discriminating between two virtual flower types. Each symbol denotes the average performance of one individual bee under one experimental condition. When targets were rewarded with sucrose solution and distractors contained no reward (plain water) (blue symbols and black regression line), bees investing more time made more accurate choices. When distractors were penalized with bitter quinine solution (red symbols and orange regression line), all bees improved their accuracy. Blue arrows link the average values for individual bees under the two experimental conditions. **b**, A blue virtual flower with a bumblebee imbibing sucrose solution from a Plexiglas platform.



a random choice condition ($\chi^2=8.73$; d.f. = 1; $P=0.031$). Between individuals, there was a strong correlation between decision time and accuracy ($r_s=0.963$; $n=10$; $P=0.00007$; Fig. 1a). The more time that an individual bee invested, the more accurate were its choices, whereas bees that made rapid choices were more error-prone. However, when errors go unrewarded, the cost of visiting the wrong flower type is comparatively low. It is not clear whether low accuracy actually reflects the limits of discrimination^{1,6,7}.

We therefore introduced higher costs for making errors by penalizing incorrect choices with aversive quinine solution. Distractor flowers each bore a 10-µl droplet of 0.12% quinine hemisulphate salt in water. After a full day of training, bees were again tested individually for three foraging bouts. Under these conditions, bees improved their accuracy significantly to 83% ($z=2.84$; $n=10$; $P=0.004$; sign test) at the expense of longer response times ($z=2.21$; $n=10$; $P=0.027$). Between bees, the correlation between time and accuracy remained significant ($r_s=0.723$; $n=10$; $P=0.018$). There was also a correlation between performance of bees in the two experiments, in terms of both accuracy ($r_s=0.951$; $n=10$; $P=0.00023$) and speed ($r_s=0.699$; $n=10$; $P=0.024$).

These results show that fast and error-prone bees in the first experiment remained fast and error-prone in the second experiment, whereas slower bees were consistently more accurate. The improvement in performance was not simply an effect of prolonged training: when the quinine penalties were

removed, accuracy fell to the same level as in the first experiment (average 61.4%).

We show that, as in humans⁸, accuracy of choice in bees depends on how much time is allocated to solving the task. Thus, whenever accuracy is quantified in discrimination tests on animals, response time should also be measured⁹, and the possibility of speed–accuracy trade-offs evaluated. Even individual insects vary in their reluctance to make errors.

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Endocrinology

Bone adaptation requires oestrogen receptor- α

The strain imposed by mechanical loading on bone tissue normally stimulates a response by bone cells that results in an adjustment of bone architecture that enables the bone to withstand reasonable loads. But it is unclear why this process should become less effective in some 50 per cent of postmenopausal women, who suffer fractures as a result¹. Here we show that bone *in vivo* undergoes an adaptive response to loading that is less effective in the absence of the α -form of the oestrogen receptor (ER- α) and that osteoblast-like cells require ER- α to proliferate in response to mechanical strain *in vitro*. As ER- α expression in osteoblasts and osteocytes depends on oestrogen concentration^{2–5}, a failure to maintain bone strength after the menopause might be due to a reduction in the activity of ER- α in bone cells, thereby limiting their anabolic response to mechanical loading and allowing a loss of bone tissue comparable to that associated with disuse.

We investigated bone's osteogenic response to loading in mice lacking functional ER- α . The normal peak locomotor strains (0.0026; length change as a proportion of original length) and strain rates (0.1 s⁻¹) in the mouse ulna shaft were determined from surgically implanted strain gauges. We then loaded the ulnae of 20–24-week-old skeletally mature female mice through their olecranon and flexed carpus for three days a week for two weeks⁶. Each loading session comprised 40 repetitions of a 3.4-newton axial compressive load, which engendered peak strains (0.0028) and maximum strain rates (0.1 s⁻¹) within the high physiological range.

In mice with normal ER- α function (ER α +/- mice), this loading regimen stimulates sufficient new bone formation on the periosteal and endosteal surfaces to increase the cortical area at the midshaft by 8 $\pm$ 0.8% (P < 0.001; Fig. 1a). In their ER α -/- littermates, however, this response was diminished threefold (2.4 $\pm$ 0.4%; P < 0.001; Fig. 1b, c).

A single period of dynamic strain applied to monolayer cultures of osteoblast-like cells stimulates their proliferation, a response that is inhibited by ER blockers and increased by transfection with additional ER- α (ref. 7). We derived separate primary cultures of osteoblast-like cells from the ulnae of ER α -/- and ER α +/- littermates and exposed them to a single 10-min period of mechanical strain (600 cycles, 1 Hz, 0.0034). Over the next 24 h, the number of ER α +/- cells increased by 58 $\pm$ 34%, P = 0.050, whereas the number of ER α -/- cells did not increase. ER α -/- mice have fully functional oestrogen receptors of the β -form, indicating

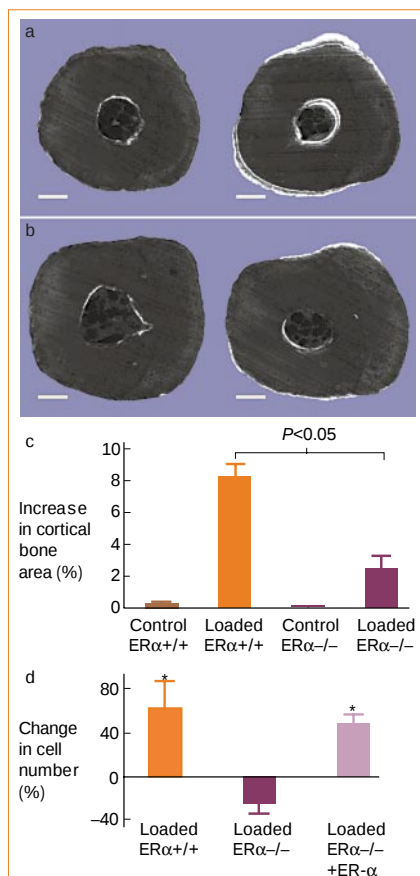


Figure 1 Absence of ER- α limits bone's adaptive response to mechanical loading. **a, b**, Transverse sections from control (left) and loaded (right) ulnae from ER α +/- (n = 7) (**a**) and ER α -/- (n = 8) (**b**) mice. New bone formation is labelled with fluorochrome calcein (shown in white), given on days 3 and 12 of the 2-week experiment. Scale bars, 100 μ m. **c**, In ulnae from ER α -/- mice, the adaptive increase in cortical bone area in response to loading was only one-third of that in ulnae from ER α +/- littermates. **d**, Mechanical strain stimulates proliferation in osteoblast-like cells derived from ER α +/- mice but not from ER α -/- mice. Transfecting osteoblast-like cells derived from ER α -/- mice with competent human ER- α confers strain-induced proliferative responsiveness in comparison with non-strained controls (asterisk denotes P $\leq$ 0.05; in five experiments with three cultures per experiment, transfection efficiency was 20 $\pm$ 2% using 'Effectene' (Qiagen), as assessed by β -galactosidase expression in 1,057 cells).

that ER- β does not compensate for incompetent ER- α in this response. However, a proliferation response to strain was conferred on ER α -/- cells by transfecting them with a functional human wild-type ER- α expression vector (pRST7-ER; ref. 8) (Fig. 1d).

These results obtained *in vivo* and *in vitro* indicate that strain-related responses by differentiated cells of the osteoblast lineage require ER- α activity. This might explain why postmenopausal women no longer maintain adequate bone mass — their bone cells are less responsive to mechanical stimulation owing to decreased ER- α activity.

The oestrogen receptor is the ancestral steroid receptor⁹, with one of its possible early reproductive functions being to induce

skeletal remodelling to release calcium for egg-laying or embryonic development. The strain-sensitive mechanisms that now enable mammalian and avian skeletons to adapt to load-bearing might have developed later, exploiting this receptor's ability to influence bone (re)modelling activity.

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COMMUNICATIONS ARISING

Metallurgy

Stainless-steel corrosion and MnS inclusions

The pitting corrosion of stainless steels that occurs in environments containing chloride usually initiates at manganese sulphide particles in the steel. Ryan *et al.*¹ describe the presence of a wide chromium-depleted zone around MnS inclusions in a stainless steel (316F grade), suggesting that pitting could be triggered by an attack on these chromium-depleted zones instead of at the MnS inclusions themselves^{2–4}. Here we investigate several different stainless steels, including the same 316F sample used by Ryan *et al.*¹, and find no evidence of chromium-depleted zones in any of them. Further analysis is evidently needed to clarify the mechanism of pitting in these materials.

Figure 1a shows a dark-field transmission electron microscope (TEM) image of a sulphide inclusion in the 316F sample used by Ryan *et al.* (kindly provided by D. E. Williams), in which most of the sulphide inclusions are connected to oxides. The TEM cross-section was prepared using a focused-ion-beam tool from FEI Co., and characterized using an FEI Tecnai scanning TEM with a probe size of less than 2 nm and a step size of about 10 nm. The energy-dispersive spectroscopy (EDS) profiles shown in Fig. 1b (associated with line 1 in Fig. 1a) reveal that there is a sharp change in composition at the interface of the MnS inclusion and matrix, with no evidence of a chromium-depletion zone around the MnS inclusion.

The linescans shown in Fig. 1b are identical to those obtained from sulphide

inclusions in several different stainless steels (results not shown). Line profiles from an as-cast 316F sample and from a sample of the cast 316F annealed at 1,100 °C also showed the same sharp boundaries. The as-cast and annealed structures should provide the opportunity for chromium to segregate to the sulphide and cause depletion in the matrix⁵, but this did not occur. Similar sharp boundaries were also evident at MnS inclusions in a standard commercial stainless steel (grade 304), indicating the absence of a chromium-depleted zone. Secondary-ion mass spectrometry (SIMS) mapping performed at MnS inclusions on polished surfaces of the 304 alloy and the 316F sample used by Ryan *et al.* also revealed no evidence of a chromium-depleted zone.

Figure 1c shows the results of a scan made along the line labelled 2 in Fig. 1a, traversing the sulphide, through the oxide, and then into the matrix. The chromium concentration is lower in the oxide than in the matrix, and even lower in the sulphide. The resulting chromium profile appears to have a chromium-depleted region, but this is really the effect of the oxide particle. The iron content in the oxide decreases more than the chromium content relative to the bulk concentration, resulting in a higher chromium/iron concentration ratio in the oxide. SIMS measurements at these particles showed that the Cr/Fe-ion yield ratio was similar or slightly higher in the oxides relative to the matrix. We therefore consider that the depleted zone described by Ryan *et al.* cannot be due to the effects of oxides adjoining sulphide particles.

Figure 1d shows the normalized ratio of raw EDS data for the linescan from the sulphide to the matrix in Fig. 1b. (This is analogous to the SIMS data shown in Fig. 1a in ref. 1.) There is little scatter and no evidence of a depleted zone near the sulphide (Fig. 1d). There is a sharp transition to the higher Cr/Fe ratio inside the sulphide particle.

In contrast to the findings of Ryan *et al.*¹, our evidence from high-resolution scanning TEM and SIMS mapping has failed to reveal the presence of chromium-depletion zones around MnS inclusions in any of the steels we investigated, at least within a nanometre-sized region.

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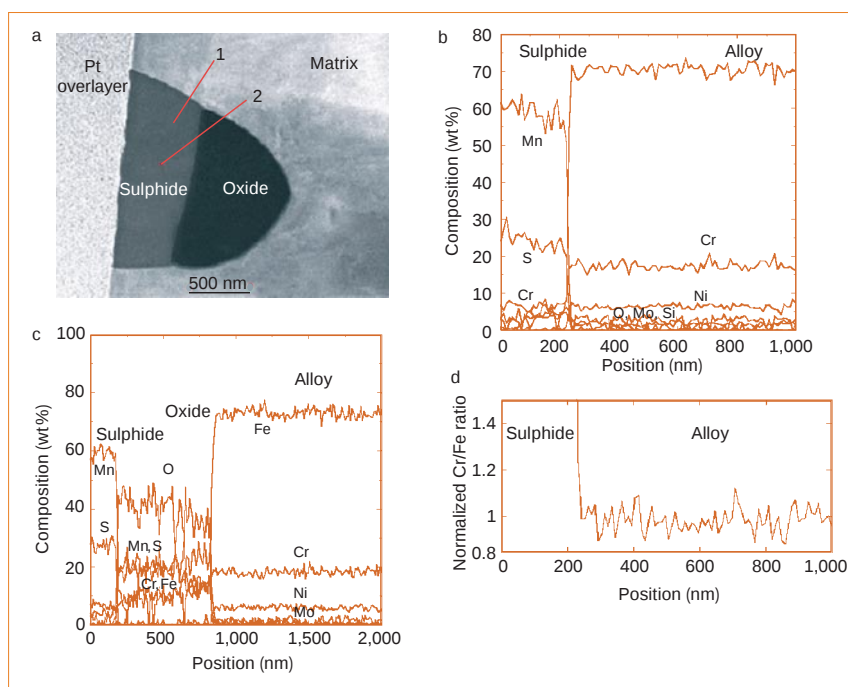


Figure 1 Analysis of composition near an MnS inclusion by nano-EDS profiling. **a**, Dark-field image in the transmission electron microscope of a typical mixed sulphide/oxide particle in the 316F stainless-steel sample used by Ryan *et al.*¹. The lines labelled 1 and 2 indicate the locations of the energy-dispersive spectroscopy (EDS) scans shown in **b** and **c**, respectively. **d**, Ratio of Cr/Fe signals from raw EDS data for the linescan shown in **b**. The ratio has been further normalized to unity to match the form of Fig. 1a in ref. 1.

Ryan *et al.* reply — Frankel *et al.* find no depleted zone adjacent to MnS inclusions in stainless steels, which contradicts our results¹ and raises important issues about pitting mechanisms. We have also used high-resolution electron microscopy and secondary-ion mass spectrometry mapping to study these systems, and our results are similar to those of Frankel *et al.*, giving a consistently large scatter in the analysis. With SIMS mapping in particular, we find that the flux required to generate a significant signal means that too much material has to be removed; in addition, the sputter rate of the inclusion is higher than that of the matrix, and sulphur ‘smears’ are observed that blur the interfaces. We conclude that this method is not useful in these systems.

By using the SIMS probe¹, we were able to analyse 25 inclusions within a reasonable time — in contrast to transmission electron microscopy, which involves complex sample preparation. Of these inclusions, some showed a large scatter in the data, and sputtering of the inclusion complicated interpretation of the results in a few other cases. Some 20% of the inclusions showed no depletion, consistent with the observations of Frankel *et al.*, but over 20% showed the type of depletion we reported earlier¹. As with all pitting studies, there is a strong statistical consideration to be taken into account — although the initiation of pitting corrosion is confined to sulphide inclusions, not all inclusions nucleate pits. We are working to develop a method that correlates inclusions that have chromi-

um depletion with subsequent pit nucleation.

Frankel *et al.* show an inclusion connected to an oxide particle. We did not analyse sites that appeared to be multiple or connected inclusions to avoid complications arising from such oxide particles². The inclusion was always sampled to ensure that it was a sulphide particle.

As we pointed out¹, the final attack on the steel that leads to an observable pit is triggered through dissolution of the inclusion. The mechanism by which this unusual dissolution occurs is the key step in the chain of events. There is further evidence indicating that the interface between inclusion and matrix is critical^{3,4}, and that there may be several precursor stages in which the electrochemical current is small^{5,6}, and we have directly investigated these aspects of corrosion.

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Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function

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The motility and morphogenesis of endothelial cells is controlled by spatio-temporally regulated activation of integrin adhesion receptors, and integrin activation is stimulated by major determinants of vascular remodelling. In order for endothelial cells to be responsive to changes in activator gradients, the adhesiveness of these cells to the extracellular matrix must be dynamic, and negative regulators of integrins could be required. Here we show that during vascular development and experimental angiogenesis, endothelial cells generate autocrine chemorepulsive signals of class 3 semaphorins (SEMA3 proteins) that localize at nascent adhesive sites in spreading endothelial cells. Disrupting endogenous SEMA3 function in endothelial cells stimulates integrin-mediated adhesion and migration to extracellular matrices, whereas exogenous SEMA3 proteins antagonize integrin activation. Misexpression of dominant negative SEMA3 receptors in chick embryo endothelial cells locks integrins in an active conformation, and severely impairs vascular remodelling. *Sema3a* null mice show vascular defects as well. Thus during angiogenesis endothelial SEMA3 proteins endow the vascular system with the plasticity required for its reshaping by controlling integrin function.

In vertebrate embryos, development of a properly patterned network of blood vessels allows efficient transport of oxygen and nutrients to tissues so that they can correctly grow, develop and function¹. The mature vascular tree is characterized by a hierarchical organization of large and small vessels. This typical pattern emerges because of a morphogenetic process known as angiogenesis, in which the homogeneously sized primary capillary plexus, previously formed by vasculogenesis, undergoes extensive remodelling. During angiogenesis, an array of environmental cues guides endothelial cells (ECs) by dynamically regulating their adhesive interactions with the extracellular matrix (ECM)¹.

Integrin heterodimers are primary ECM receptors, and several integrins have been implicated in angiogenesis². The most extensive body of data links blood vessel development to $\alpha\text{v}\beta 3$ integrin—a receptor for both fibronectin (FN) and vitronectin (VN)—and $\alpha\text{v}\beta 5$ integrin, a VN receptor. Blockade of $\alpha\text{v}\beta 3$ (refs 3, 4) or $\alpha\text{v}\beta 5$ (ref. 5) integrins with antagonists disrupts tumour and experimental angiogenesis. In contrast, genetic experiments showed that successful vasculogenesis and angiogenesis depend on FN (ref. 6) and its main receptor, $\alpha 5\beta 1$ integrin⁷, but not on αv integrins⁸. This discrepancy has been recently resolved by reports suggesting that $\alpha\text{v}\beta 3$ integrin might be an environmental biosensor; when unligated, for example, in the presence of $\alpha\text{v}\beta 3$ antagonists, it triggers an 'integrin-mediated death' response that would prevent undesirable vessel formation on non-angiogenic ECM (ref. 9). Integrins can exist in different functional states with respect to their affinity or avidity for ECM proteins, and regulation of integrin activation is crucial for their biological functions¹⁰. High affinity integrins are recruited to the leading edge of migrating ECs where they promote new adhesions to support directed cell motility¹¹.

Moreover, regulated activation of integrins is essential for embryonic morphogenesis¹², and prominent determinants of vascular remodelling, such as fluid shear stress¹³ and angiogenic growth factors^{14,15}, activate integrin adhesive function. On the basis of these observations, we speculated that dynamic control of integrin function could be one of the molecular mechanisms underlying the plasticity necessary for angiogenic remodelling, and that inhibitors of integrins might be required to counterbalance the effects of activators.

In an attempt to identify negative regulators of integrin function, we hypothesized that EC integrins could be inhibited by SEMA3 proteins, a family of secreted repellents for navigating axons whose receptors are expressed on the surface of ECs as well¹⁶. Indeed, the prototypic vertebrate semaphorin, SEMA3A (ref. 17) can inhibit EC motility¹⁸, and genetic ablation of neuropilin 1 (Npn1, also known as Nrp1), which is the ligand-binding moiety of the SEMA3A receptor complex, causes vascular defects in mouse embryos¹⁹.

SEMA3 autocrine signalling in angiogenic ECs

To assess the potential role of SEMA3 proteins as *in vivo* regulators of EC function, we first investigated the expression of *Sema3A* in animal models of developmental and experimental angiogenesis. In embryonic day 10 (E10) mice, a thin layer of *Sema3A* immunoreactivity was evident in ECs facing the luminal side of dorsal aorta both in the trunk (Fig. 1a, b, asterisk) and in the head of wild type (Fig. 1e, f), but not of *Sema3A* null (Fig. 1g, h), embryos. Factor VIII (Fig. 1c) and α -smooth muscle (α -SM) actin (Fig. 1d) immunostainings on adjacent sections confirmed that *Sema3A* positive cells (Fig. 1b) were ECs and not SM cells. At E12.5, ECs of small perineural blood vessels (Fig. 1f, arrowhead) that sprout to invade

the brain parenchyma expressed *Sema3A*. To determine whether *Sema3A* synthesis by ECs was restricted to vascular development, we examined the vascular channels formed in vascular endothelial growth factor (VEGF)-A-loaded Matrigel plugs in adult mice. Immunohistochemical analysis of plugs removed 10 days after

implantation showed that ECs of neo-vessels sprouting into the Matrigel synthesized *Sema3A* (Fig. 1i). Moreover, in the chick embryo chorioallantoic membrane (CAM) assay, an *in vivo* model widely used to study neo-angiogenesis, *Sema3A* was expressed by ECs (Fig. 1k) but not by SM cells (Fig. 1l) of neo-vessels surrounding VEGF-A-treated gelatin sponges implanted on CAM (Fig. 1k, l, arrowhead). No *Sema3A* expressing blood vessels are detectable around saline soaked gelatin sponges (Fig. 1j). Notably, *Sema3A* was not synthesized by ECs of quiescent blood vessels, either in untreated adult mice or in unstimulated chick CAMs (not shown). Thus, *Sema3A* is produced by angiogenic ECs during vascular development and experimental angiogenesis.

We next examined *SEMA3* gene expression *in vitro* in human ECs (see Supplementary Information), and found that these cells synthesize five out of six *SEMA3* proteins as well as the ligand binding and the signal transducing subunits of *SEMA3* receptor complexes, represented by *Npn1* and *Npn2* (which is also known as *Nrp2*) (ref. 16) and the type A plexins (*PlexA*), respectively¹⁷. Synthesis of *SEMA3* by unstimulated ECs is probably due to the fact that, when grown *in vitro*, ECs become activated and express molecules usually induced *in vivo* by angiogenic factors²⁰.

Autocrine *SEMA3* proteins regulate integrin function

Tyrosine phosphorylation is a key regulatory mechanism involved in the formation and turnover of ECM adhesions²¹. We therefore analysed the spatial relationships between endogenous *SEMA3A* and phosphotyrosine-containing adhesive structures²¹ of ECs spreading on FN (Fig. 2). At the cell periphery *SEMA3A* either colocalized or displayed a perfect alternate staining with small dot-like phosphotyrosine-containing adhesive complexes, known as focal complexes²¹ (Fig. 2). Focal complexes are dynamic and rapidly turned-over adhesive structures enriched with high affinity integrins^{11,21} that generate transient pulses of propulsive traction forces during cell migration²². Dismantling old focal complexes and shifting their assembly to a new protrusive region could support rapid reorientation in response to environmental cues²². The fact that chemorepulsive *SEMA3A* colocalizes with, flanks, or surrounds focal complexes within the same cell (Fig. 2) suggests dynamic transient association and sorting between *SEMA3A* and integrin-containing adhesive structures; this could be a means of generating adhesive plasticity without causing complete de-adhesion. Therefore, we hypothesized that during cell migration autocrine loops involving *SEMA3* regulate EC responsiveness to directional cues by inhibiting integrin function at focal complexes. This could be particularly crucial for an efficient EC reorientation during vascular remodelling. To directly test this hypothesis, we studied adhesion and spreading on FN and VN of ECs in which *SEMA3* autocrine loops were disrupted by retroviral transduction with vectors carrying dominant negative (DN) constructs of *PlexA1*²³ or *Npn1* that inhibit the activity of both *Npn1* and *Npn2* in *SEMA3* protein binding, but not that of *Npn1* in binding the angiogenic factors VEGF-A₁₆₅ and placental growth factor 2 (PlGF2; see Supplementary Information).

Compared to wild-type ECs transduced with GFP, ECs expressing either *Npn1*-DN (*Npn1*-DN ECs) or *PlexA1*-DN (*PlexA1*-DN ECs) adhered and spread more efficiently on FN (Fig. 3a) and VN (Fig. 3b) at different substrate concentrations. The increase in adhesion was integrin-dependent, since it was inhibited on FN (Fig. 3c) by the monoclonal antibodies (mAbs) JBS5 and LM609, which block $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins respectively, and on VN (Fig. 3d) by mAb LM609. Furthermore, addition of exogenous *SEMA3A* (Fig. 3e, f) or *SEMA3F* (not shown) inhibited adhesion of wild-type ECs to substrates with different concentrations of FN (Fig. 3e) and VN (Fig. 3f). Adhesion of ECs expressing *PlexA1*-DN or *Npn1*-DN to FN (Fig. 3g) or VN (Fig. 3h) was not inhibited by exogenous *SEMA3A*, and the $\beta 1$ integrin-stimulating antibody HUTS-4 overcame *SEMA3A* inhibition of wild-type EC adhesion

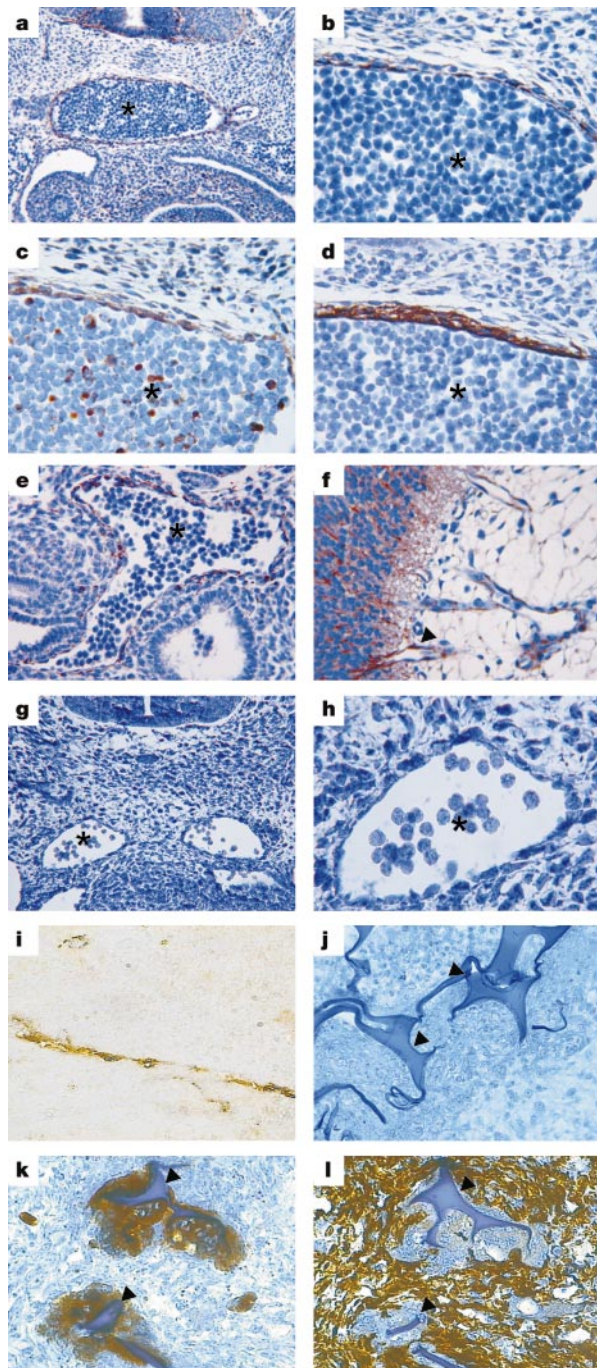


Figure 1 *SEMA3A* expression during vascular development and experimental angiogenesis. **a, b, e, f**, In E10 mouse embryo, *Sema3A* is present in spinal cord (**a**), and in dorsal aorta ECs (asterisk) in trunk (**a, b**), and head (**e**), but not in α -SM actin positive cells of adjacent sections (**d**). *Sema3A* positive cells also stain for factor VIII (**c**). In E12.5 embryo, *Sema3A* is detected in ECs of perineural small blood vessels (arrowhead) (**f**). **g, h**, dorsal aorta of *sema3A*^{-/-} embryos is negative. **i**, *Sema3A* is present in ECs of VEGF-A-loaded Matrigel plugs. **j-l**, In chick CAM, *Sema3A* is absent around saline-soaked gelatin sponges (**j**); *Sema3A* is expressed by ECs of neo-vessels surrounding trabeculae (arrowheads) of VEGF-A-soaked sponges (**k**), but absent in α -SM actin positive cells (**l**). **b** is a magnification of **a**; **b, c, d** are adjacent; **h** is a magnification of **g**; and **k** and **l** are adjacent.

to FN (Fig. 3g). VEGF-A and basic fibroblast growth factor (bFGF) are well-established activators of endothelial integrin function both *in vivo*^{3,5,24,25} and *in vitro*¹⁴. Exogenous SEMA3A (Fig. 3i) inhibited both VEGF-A- and bFGF-enhanced adhesion of ECs to FN and VN, further suggesting a physiological role for SEMA3 proteins in regulating integrin-mediated EC adhesion.

Next, we tested whether the observed negative regulation of integrin function by SEMA3 proteins results from inhibition of integrin affinity for ECM ligand(s). We monitored affinity modulation of $\alpha v\beta 3$, analysing by fluorescence-activated cell sorting (FACS) the binding to ECs of the soluble monovalent ligand mimetic WOW-1 Fab, which reacts selectively with activated $\alpha v\beta 3$ integrin^{14,26}. SEMA3A completely inhibited the increase in WOW-1 Fab binding to ECs induced by either VEGF-A or bFGF (Fig. 3j). Thus, autocrine signalling by SEMA3 proteins exerts an inhibitory

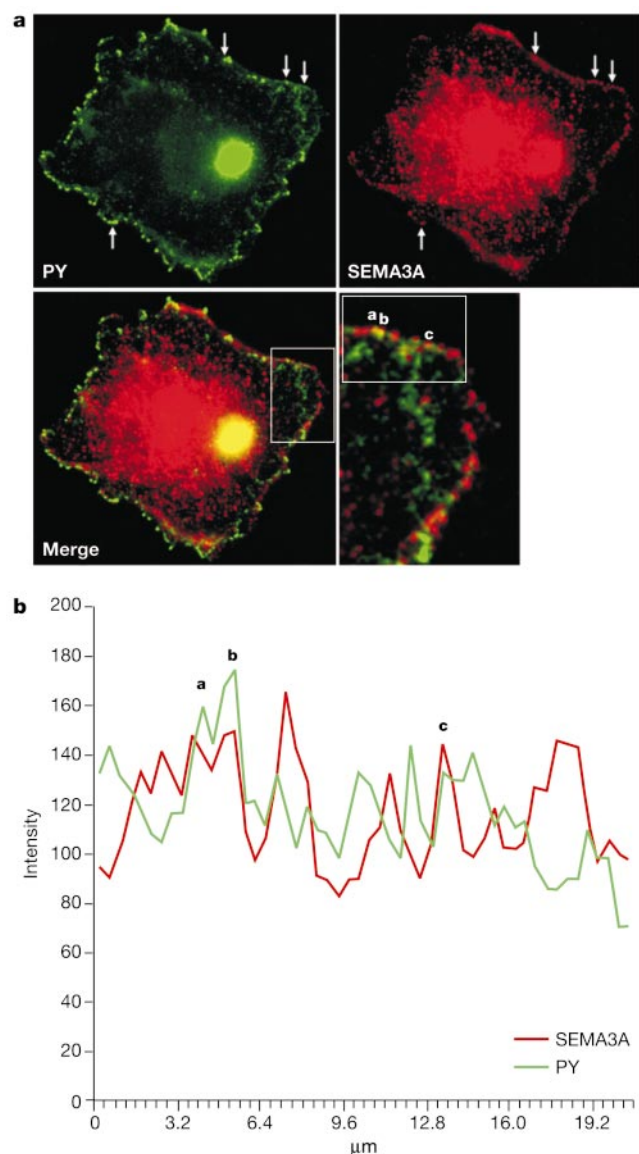


Figure 2 Relationships between endogenous SEMA3A and nascent focal complexes. **a**, ECs adhering on FN stained with anti-phosphotyrosine (top left; PY, green) and anti-SEMA3A (top right; SEMA3A, red). Arrows indicate areas of colocalization. Bottom right panel is an enlargement of the bottom left panel boxed region, where colocalization (a, b, c lettering) and alternate staining areas are evident at cell edge. **b**, Fluorescent intensity of the cell edge shown in the bottom right panel boxed area (**a**). X-axis origin corresponds to the left margin of the boxed region. Peaks a, b, c correspond to colocalization areas shown in right bottom panel of **a**.

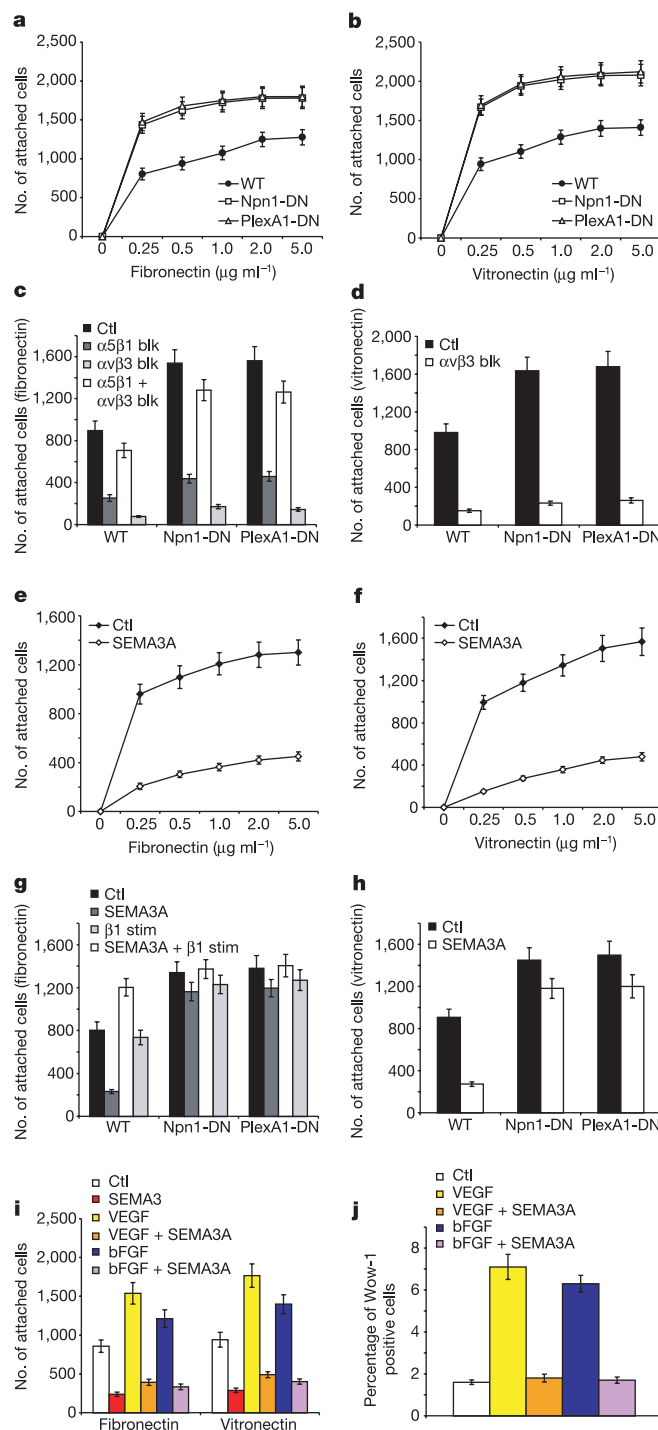


Figure 3 SEMA3 proteins regulate EC adhesion. **a–d**, Endogenous SEMA3 proteins regulate integrin-mediated adhesion. **a**, **b**, EC adhesion to FN (**a**) or VN (**b**) at various concentrations. **c**, **d**, Cell adhesion on FN (**c**) and VN (**d**) in absence (Ctl) or presence of mAbs JBS5 blocking $\alpha 5\beta 1$ ($\alpha 5\beta 1$ blk), LM609 inhibiting $\alpha v\beta 3$ ($\alpha v\beta 3$ blk), or both of them ($\alpha 5\beta 1 + \alpha v\beta 3$ blk). **e–j**, Exogenous SEMA3A inhibits integrin activation. **e**, **f**, EC adhesion to FN (**e**) or VN (**f**) at various concentrations in absence (Ctl) or presence of SEMA3A. **g**, Cell adhesion on FN in absence (Ctl) or presence of SEMA3A, with or without $\beta 1$ integrin stimulatory ($\beta 1$ stim) mAb HUTS-4. **h**, Cell adhesion on VN in absence (Ctl) or presence of SEMA3A. **i**, Stimulation of EC adhesion to FN or VN by VEGF or bFGF in absence or presence of SEMA3A. **j**, WOW-1 Fab binding to ECs stimulated with VEGF or bFGF in absence or presence of SEMA3A.

effect on integrin-mediated adhesion to different provisional ECM components, such as FN and VN, which are permissive for tissue vascularization². Moreover, SEMA3 proteins appear to exert their regulatory effect on EC adhesion by directly inhibiting ECM ligand recognition by integrins.

Because regulated cell locomotion on a permissive ECM is essential for angiogenesis, we next investigated the role of SEMA3 autocrine signalling on directed migration of ECs in response to substratum-bound FN and VN. In a Transwell system, both Npn1-DN and PlexA1-DN expressing ECs showed a higher locomotion towards FN (Fig. 4a) and VN (Fig. 4b) at different substrate concentrations. Enhanced migration towards FN was inhibited by the $\alpha 5 \beta 1$ blocking mAb JBS5 and the $\alpha v \beta 3$ inhibiting mAb LM609 (Fig. 4c). The latter inhibited increased migration towards VN as well (Fig. 4d). Accordingly, exogenous SEMA3A (Fig. 4e, f) or SEMA3F (not shown) inhibited migration of wild-type ECs towards different FN (Fig. 4e) and VN (Fig. 4f) concentrations. Migration of PlexA1-DN and Npn1-DN ECs towards FN (Fig. 4g) or VN (Fig. 4h) was not inhibited by exogenous SEMA3A; the inhibitory effect on wild-type EC migration to FN was reversed by the $\beta 1$ integrin-stimulating antibody HUTS-4 (Fig. 4g).

To study directly how autocrine loops of SEMA3 proteins control EC motility, cell migration was measured by time-lapse video microscopy, tracking motility patterns of individual ECs plated on FN. In this macroscopically homogeneous environment, cell polarization arises from spatial stimulus gradients caused by microscopic non-uniformities²⁷. Expression of DN SEMA3 receptors did not significantly influence EC migration speed (mean migration speed $\pm$ s.d.: wild-type, $31 \pm 5 \mu\text{m h}^{-1}$; PlexA1-DN, $34 \pm 7 \mu\text{m h}^{-1}$; Npn1-DN, $33 \pm 7 \mu\text{m h}^{-1}$), but resulted instead in a higher directional persistence of cell migration as visualized by

wind-rose plots; while wild-type ECs (Fig. 4i, upper left panel) moved in random directions, both Npn1-DN (Fig. 4i, upper right panel) and PlexA1-DN ECs (Fig. 4i, lower left panel) tended to continue to migrate in a particular direction. Differences in migration patterns were quantified by comparing the ratios of the shortest direct distance from the starting point of each recording to the end point (D), to the total distance traversed by the cell (T)²⁸. The directional persistence (D/T ratio) of Npn1-DN and PlexA1-DN ECs was respectively 2.4- and 2.6-fold higher than that of wild-type ECs (Fig. 4i, lower right panel). Thus, SEMA3 autocrine loops negatively regulate EC migration rate and facilitate cell reorientation by inhibiting integrin function.

Endothelial SEMA3 proteins control vascular morphogenesis

Since angiogenic remodelling during vascular morphogenesis is an event in which the adhesive state of ECs undergoes modulation, we sought to test whether SEMA3 autocrine signalling could be required for the execution of this complex morphogenetic process, making use of the same DN constructs for SEMA3 receptors that we have shown to affect EC integrin function *in vitro*. In the chick embryo, between stages 17 and 23, the homogeneous primary capillary plexus of the head undergoes dramatic reshaping into a hierarchically organized vascular system^{29,30}. In stage 19 embryos, we found that blood vessels of the perineural primary capillary plexus, which surrounds the forebrain, actively synthesize *Sema3A* (Fig. 5a). Therefore, we disrupted the autocrine *Sema3* loops in the developing head vasculature by expressing DN constructs of SEMA3 receptors using the replication-competent retrovirus RCAS-BP(A) (ref. 31). At stage 17, the vascular plexus of the head is a major site of EC proliferation³². We found that injecting RCAS-BP(A) retrovirus carrying GFP into the blood stream of stage 17 embryos results in an

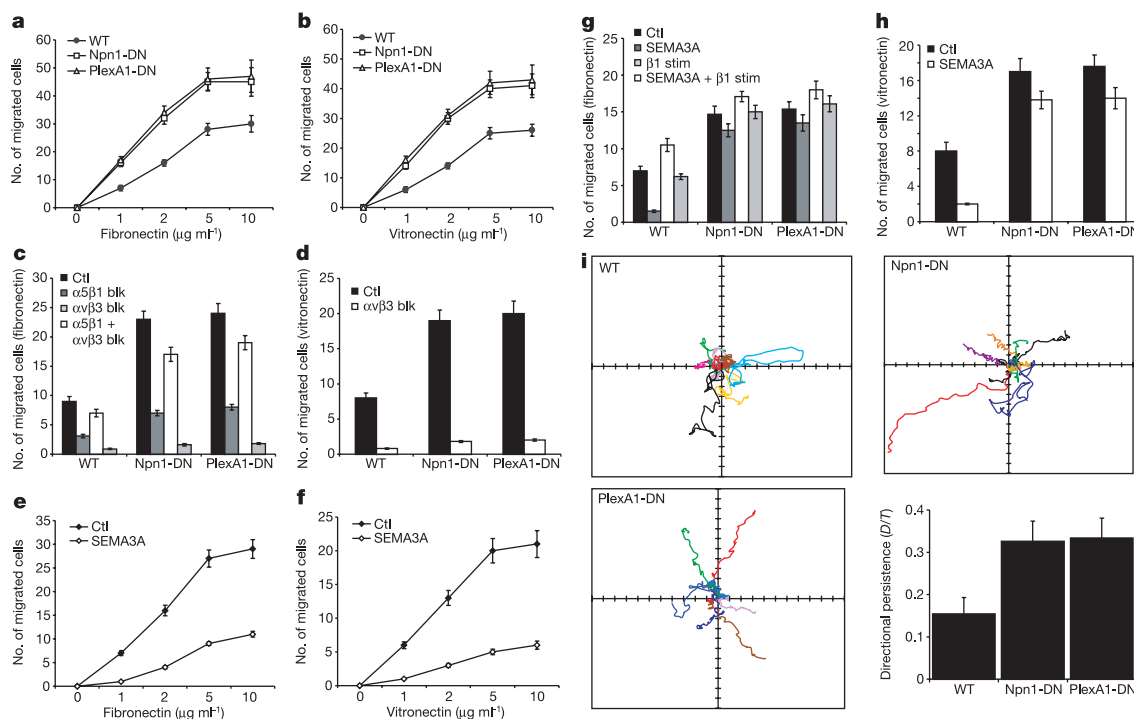


Figure 4 SEMA3 proteins regulate EC migration towards ECM. **a–d**, Endogenous SEMA3 proteins regulate integrin-mediated cell migration. **a, b**, EC migration towards FN (**a**) or VN (**b**) at various concentrations. **c, d**, EC migration towards FN (**c**) or VN (**d**) in absence (Ctl) or presence of mAbs JBS5 blocking $\alpha 5 \beta 1$ ($\alpha 5 \beta 1$ blk), or LM609 inhibiting $\alpha v \beta 3$ ($\alpha v \beta 3$ blk), or both of them ($\alpha 5 \beta 1 + \alpha v \beta 3$ blk). **e–h**, Exogenous SEMA3A inhibits integrin-mediated cell migration. **e, f**, EC migration towards FN (**e**) or VN (**f**) at various concentrations in absence (Ctl) or presence of SEMA3A. **g**, EC migration towards FN in

absence (Ctl) or presence of SEMA3A, with or without $\beta 1$ integrin stimulatory ($\beta 1$ stim) mAb HUTS-4. **h**, EC migration towards VN in absence (Ctl) or presence of SEMA3A. **i**, Directional motility is SEMA3-mediated. Wind-rose plots of wild-type (WT; top left), Npn1-DN (top right), and PlexA1-DN (bottom left) EC paths tracked over 4 h and replotted such that all paths start from origin. Bottom right panel, black bars represent directional persistence (D/T).

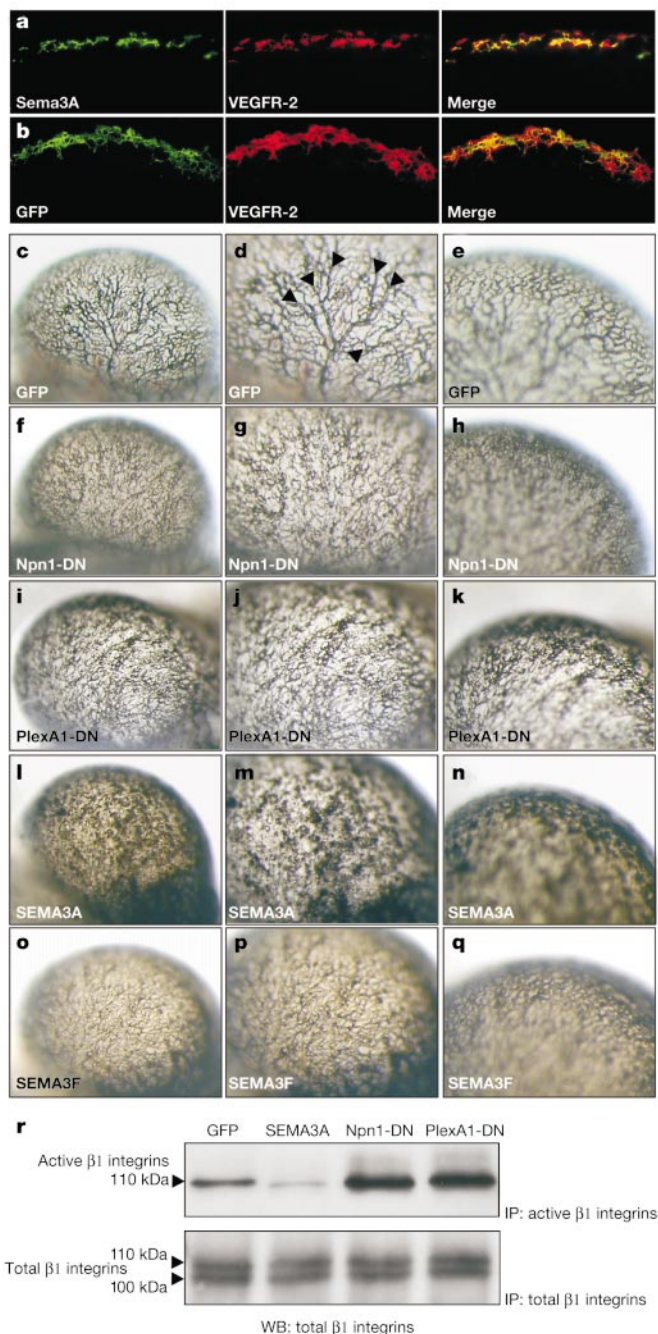


Figure 5 SEMA3 proteins are required for chick embryonic vascular remodelling. **a**, In stage 19HH embryos, SEMA3A (left) is present in perineural vascular plexus ECs (VEGFR-2 positive, centre) surrounding forebrain. SEMA3A and VEGFR-2 colocalize (right). **b**, GFP (left) is expressed by perineural capillary plexus ECs (VEGFR-2 positive, centre) of 23HH embryos infected with RCAS-BP(A)-GFP. GFP and VEGFR-2 colocalize (right). **c–q**, RCAS-BP(A)-Npn1-DN, -PlexA1-DN, -SEMA3A, and -SEMA3F cause remodelling defects of head vasculature. **c–e**, India-ink-injected head mature vascular bed of 23HH embryos infected with RCAS-BP(A)-GFP displays large blood vessels in the central region (**c**, arrowheads in **d**) and a peripheral network of smaller blood vessels (**e**). **f–q**, Head vasculature of RCAS-BP(A)-Npn1-DN (**f–h**), -PlexA1-DN (**i–k**), -SEMA3A (**l–n**), -SEMA3F (**o–q**) infected embryos is characterized by the absence of major blood vessels (**f, g, i, j, l, m, o, p**) and by an immature peripheral vascular network (**h, k, n, q**). **d** and **e**, **g** and **h**, **j** and **k**, **m** and **n**, **p** and **q** are respectively magnifications of **c, f, i, l**, and **o, r**. SEMA3 proteins regulate integrin activation during vascular development. Head lysates of chick embryos infected with GFP, SEMA3A, Npn1-DN and PlexA1-DN were immunoprecipitated (IP) with TASC/9D11 mAb (active chicken $\beta 1$ integrins) or with V2E9 mAb (total $\beta 1$ integrins) and blotted with V2E9. $\beta 1$ integrin subunit appears as immature 100-kD and mature 110-kD bands.

efficient infection of ECs of perineural vascular network (Fig. 5b) of the head by the time of its angiogenic remodelling. The control virus ($n = 37$) had no effect on head vascular morphogenesis; stage 23 embryos were characterized by the appearance of large-diameter blood vessels (Fig. 5c, d arrowheads), which were connected to a highly branched and intricate network of smaller blood vessels (Fig. 5e). In contrast, both RCAS-BP(A)-Npn1-DN ($n = 39$; Fig. 5f–h) and RCAS-BP(A)-PlexA1-DN ($n = 36$; Fig. 5i–k) caused defective angiogenesis. The head vascular bed of stage 23 embryos infected with DN SEMA3 receptors was not remodelled into large-diameter blood vessels (Fig. 5f, g, i, j) and appeared arrested at the primary plexus stage (Fig. 5h, k).

To verify whether during vascular remodelling different SEMA3 proteins act at different sites, we overexpressed either SEMA3A or SEMA3F in ECs of the perineural vascular network of the head of developing chick embryos. Expression of RCAS-BP(A)-SEMA3A ($n = 31$; Fig. 5l–n) and RCAS-BP(A)-SEMA3F ($n = 34$; Fig. 5o–q) resulted in a dramatic inhibition of vascular remodelling, whose patterns are very similar to each other and to those observed after overexpression of DN SEMA3 receptors. Hence, during development, tight regulation of SEMA3 activity is required for proper vascular remodelling. Since *in vitro* SEMA3 proteins exert their effects on ECs by regulating integrin function, we investigated whether vascular remodelling defects observed in chick embryos after EC infection with DN SEMA3 receptors or SEMA3 could be related to modulation of integrin function. Active chick $\beta 1$ integrins were immunoprecipitated from head tissue extracts of infected chick embryos with the conformation-specific mAb TASC^{33,34} (Fig. 5r). Retrovirus-driven overexpression of either DN SEMA3 receptors or SEMA3A respectively resulted in either hyperactivation or inhibition of $\beta 1$ integrins in the endothelium of developing chick embryos, while the content of total $\beta 1$ integrins was unaffected (Fig. 5r). Thus, suppression of integrin function by SEMA3 auto-crine loops is required for angiogenic remodelling during vascular development.

To investigate further the role of SEMA3 proteins *in vivo*, we analysed vascular development in CD-1 mouse embryos in which the *Sema3a* gene has been disrupted³⁵. Among eight homozygous E9.5 embryos from three litters, seven (87%) showed vascular defects in the head and six (75%) had abnormal trunk blood vessels. This vascular phenotype was not, however, observed in a 129/Sv background³⁶. It is well documented that the phenotypes of a mutant show variations depending on genetic background effects due to allelic variation outside the target locus³⁷; such an effect has in fact been reported for the *Sema3c* knockout mice³⁸. Partial penetrance could be a consequence of overlapping functions among SEMA3 family members, as suggested by the finding that cultured ECs express five out of six SEMA3. At E9.5, heads of heterozygous embryos contained a hierarchically organized vasculature with large-diameter branches extending from the anterior cardinal vein (Fig. 6a, white arrowheads). Cranial blood vessels of *Sema3a*^{−/−} embryos were not remodelled, and maintained a primary capillary plexus appearance (Fig. 6b). In E9.5 heterozygous embryos, the dorsal aorta (Fig. 6c, black arrowheads) and anterior cardinal vein (Fig. 6c, white arrowheads) are easily recognizable. In severely affected *Sema3a*^{−/−} mutant embryos, the dorsal aorta formed normally (Fig. 6d, black arrowheads), whereas development of the anterior cardinal vein was disrupted (Fig. 6d). Defects in the anterior cardinal vein persisted until at least E12.5 (not shown). Moreover, while the finely anastomosed network, which results from remodelling of the dorsal side of intersomitic vessels, was present in E9.5 heterozygotes (Fig. 6e), it was not present in homozygotes (Fig. 6f).

Discussion

Remodelling of the early vasculature into a mature arborized tree is a critical event for normal embryonic development. Such a vascular

reshaping is the end result of a complex series of dynamic cell-to-cell and cell-to-ECM interactions driven by fluid shear stress and angiogenic growth factors³⁹. We have shown that autocrine loops of SEMA3 chemorepellents exert an essential permissive role in the execution of this complex morphogenetic process by inhibiting integrin-mediated adhesion of ECs to the ECM, allowing the de-adhesion necessary for vascular remodelling. Autocrine secretion of *Sema3C* has also been found in SM cell precursors—that is, cardiac neural crest cells—which interact with, and help to remodel, aortic arches⁴⁰. The fact that in *Sema3c* null mice³⁸ cardiac neural crest migration is disrupted, resulting in cardiac outflow tract and aortic arch abnormalities, further points to SEMA3 autocrine signals as regulators of vascular development. Furthermore, the identification of SEMA3B⁴¹ and SEMA3F⁴² as tumour suppressor proteins working in an autocrine fashion suggests that SEMA3 autocrine loops could play key roles outside the cardiovascular system as well. Loss of heterozygosity of these genes might potentially foster deregulated tumour cell adhesion and migration.

Dynamic regulation of integrin-mediated adhesion plays a central role in cell migration^{11,21,22,27}. Our results demonstrate that inhibitory autocrine loops of SEMA3 proteins are required for continuous and subtle modulation of integrin function, as compared to an all-or-none activation. Such a fine-tuning of integrin-mediated adhesion to the ECM allows a graded control of EC migration rate and redirection during migration. The finding that endogenous SEMA3A localizes to focal complexes in spreading

ECs indicates that during cell migration the autocrine presentation of SEMA3 proteins could dynamically destabilize integrin-based cell protrusions in order to enable the formation of new ones at positions where the actual concentration of attractive guidance cues is higher. Although additional studies are required to shed light on the mechanisms by which SEMA3 proteins inhibit integrin function, here we have provided evidence that exogenous SEMA3A exerts direct control on integrin affinity for ECM ligands.

In this context, it is of interest to note that MICAL (molecule associated with CasL) was recently identified as a novel PlexinA interacting protein that mediates SEMA signaling¹⁷. CasL is a member of the Crk-associated substrate (Cas) family of adaptors that lies downstream of the focal adhesion kinase (FAK) in promoting integrin-mediated directional motility. In migrating cells, localized Rac GTPase activation promotes an actin-based pseudopodial extension that is stabilized by nascent focal complexes. New integrin contacts then promote FAK/Cas/Crk/Dock 180 guanine-nucleotide exchange factor coupling, which activates Rac and provides a positive-feedback loop maintaining membrane extension and adhesion at the leading edge⁴³. The proline-rich region of MICAL could conceivably compete with FAK for binding to the amino-terminal SH3 domain of Cas and cause FAK/Cas/Crk/Dock 180 uncoupling.

Our analysis of the developing chick embryo has revealed that both *in vitro* and *in vivo* SEMA3 proteins regulate integrin function, and that *in vivo* overexpression of either DN SEMA3 receptors or SEMA3A result both in defective vascular remodelling and altered activation of $\beta 1$ integrins. The observation that both inhibition and hyperactivation of integrin function are associated with defective blood vessel reshaping further suggests that modulation of integrin adhesive activity is mandatory for the accomplishment of the cardiovascular morphogenetic program. All the vascular defects that we observed in *Sema3a*^{-/-} embryos phenocopy in part the mutation in ephrin-B2, EphB4 and EphB2/EphB3⁴⁴. The Eph/ephrin signalling has also been shown to modulate integrin function⁴⁵. Taken together, these results suggest that repulsive cues might regulate vascular morphogenesis quite generally by modulating integrin activation. □

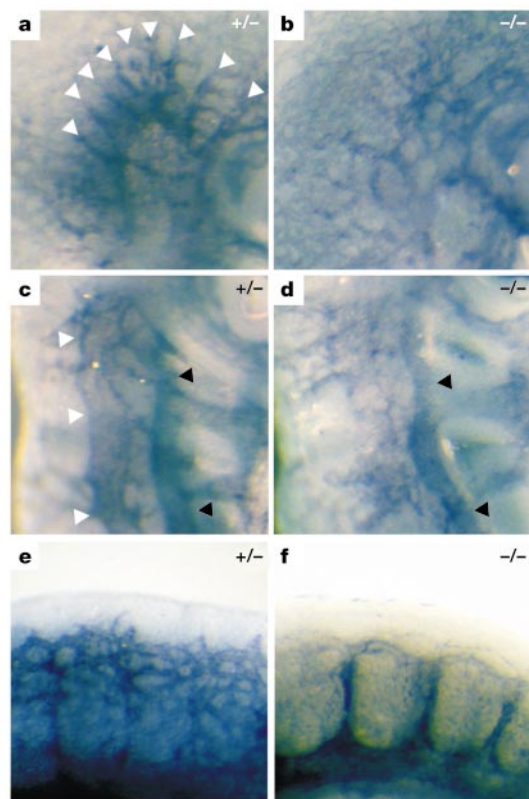


Figure 6 Vascular defects in *Sema3a*^{-/-} mouse embryos. **a–f**, Whole mount PECAM-1 stained E9.5 embryos. **a, b**, Head region (rostral up, dorsal left). Branches of anterior cardinal vein are evident in heads of *Sema3a*^{+/-} (**a**, white arrowheads) but not *Sema3a*^{-/-} embryos (**b**). **c, d**, Anterior trunk region (rostral up, dorsal left). Dorsal aorta (**c, d**, black arrowheads) is present both in *Sema3a*^{+/-} (**c**) and *Sema3a*^{-/-} embryos (**d**). Anterior cardinal vein, normally present in *Sema3a*^{+/-} embryos (**c**, white arrowheads), is disrupted in *Sema3a*^{-/-} embryos (**d**). **e, f**, Intersomitic vessels (dorsal up). The dorsal network, resulting from intersomitic vessels' remodelling in E9.5 heterozygotes (**e**), is absent in homozygotes (**f**).

Methods

Antibodies, recombinant proteins, and growth factors

Goat polyclonal anti-SEMA3A (N-15, C-17 and Q18) and Npn1 (C-19), rabbit polyclonal anti-Npn1 (H-286) and anti-VEGFR-2 (C-1158) were from Santa Cruz Biotechnology Inc. Rabbit polyclonal anti-factor VIII was from Dako. Monoclonal antibodies anti- α SM actin (1A4), anti-myc (9E10), anti-HA (HA-7) and anti-VSV (P5D4) were all from Sigma Chemical Co. MAbs HB1.1 anti-human $\beta 1$ integrins, JBS5 (blocking $\alpha 5 \beta 1$ integrin), HUTS-4 (stimulating $\beta 1$ integrins), LM609 (inhibiting $\alpha v \beta 3$ integrin), V2E9 anti-chicken $\beta 1$ integrins, and TASC9D11 anti-active chicken $\beta 1$ integrins were all from Chemicon. Anti-PECAM was from Pharmingen. WOW-1 Fab was a gift from S. Shattil (The Scripps Research Institute). Anti-GFP mAb (A-11120) was from Molecular Probes. AC-15 mAb to β -actin was from Accurate. Anti-phosphotyrosine mAb(05-321) was from Upstate Biotechnology.

Recombinant human VEGF-A₁₆₅ was from R&D Systems. bFGF was from Roche. Recombinant human PlGF2 was from RELIATech GmbH. Affinity purified recombinant human SEMA3A/Fc chimera was a gift from R&D Systems and was employed at a final concentration of 200–700 ng ml⁻¹. Human plasma FN and VN were from Sigma.

Retroviral transduction of ECs

Npn1-DN (see Supplementary Information) and PlexA1-DN (ref. 23) constructs were cloned under the CMV promoter of the retroviral expression vector PINCO, that allowed a high efficiency gene transfer in human ECs from umbilical cord veins as previously described^{46,47}.

Adhesion assay

10,000 ECs were resuspended in 0.1 ml of medium 199 with or without appropriate stimuli, plated on 96-well microtitre plates (Costar) coated with either FN or VN (0.5 μ g ml⁻¹ unless indicated otherwise), incubated for 30 min at 37 °C, washed three times with PBS, fixed in 8% glutaraldehyde, and stained with 0.1% crystal violet, 20% methanol. Before stimulation with angiogenic growth factors ECs were starved overnight.

FACS analysis

Binding of WOW-Fab was assessed by FACS as previously reported¹⁴.

Cell migration assay

30,000 ECs were seeded on the upper side of 8 μm pore filters of Transwell chambers (Costar) coated on the lower side with either FN or VN ($1 \mu\text{g ml}^{-1}$ unless indicated otherwise), and incubated in medium 199 with or without appropriate stimuli for 3 h. Cells on the upper side of the filters were then mechanically removed. ECs of the filter lower side were then fixed in 8% glutaraldehyde for 30 min and stained with 0.1% crystal violet. Before stimulation with angiogenic growth factors ECs were starved overnight.

Avian retroviral constructs and targeting

Fertilized white Leghorn chicken embryo eggs were incubated at 37 °C in a humidified incubator and staged according to ref. 48. Npn1-DN, PlexA1-DN, SEMA3A, SEMA3F and GFP gene were cloned into the RCASBP(A) plasmid as described in ref. 31. Concentrated viruses with a titre of $(6-8) \times 10^8$ pfu ml^{-1} were injected into the heart of the embryos at stage 17HH using the CellTram microinjector (Eppendorf) equipped with sterile Femtotips II pre-pulled glass capillaries (Eppendorf). Embryos at stage 23HH were processed for further analysis. Whole mount staining of blood vessels was performed by injecting intravenously India ink (Pelikan N°17).

Immunoprecipitation of active $\beta 1$ integrins from chick embryos

Heads of infected chick embryos were harvested at stage 21 or 23HH, snap frozen in liquid nitrogen, and homogenized with a motorized grinder in a lysis buffer containing Tris-HCl 50 mM pH 7.4, NaCl 150 mM, 1% Triton X-100, and protease and phosphates inhibitors (pepstatin, 50 mg ml^{-1} ; leupeptin, 50 mg ml^{-1} ; aprotinin, 10 mg ml^{-1} ; 2 mM PMSF; 500 mg ml^{-1} soybean trypsin inhibitor, 100 mM ZnCl_2). Pools of 4–5 heads were homogenized in 1 ml of lysis buffer, incubated for 1 h on wet ice, and then centrifuged at 15,000g, 15 min, 4 °C. The total protein amount was determined using the BCA protein assay reagent (Pierce). Equivalent amounts (800 μg) of protein from the same sample were immunoprecipitated for 1 h either with TASC/9D11 mAb, which recognize active chicken $\beta 1$ integrins^{33,34}, or with V2E9 mAb, reacting with total chicken $\beta 1$ integrins. Immune complexes were recovered on anti-mouse IgG-agarose (Sigma). Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without Triton, once with TBS, and then separated on 8% SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore), probed with V2E9, and detected by enhanced chemiluminescence technique (NEN Life Science Products).

Analysis of *Sema3a* null mice

Sema3a^{+/-} mice used in this study were backcrossed to CD1 mice for more than five generations and genotyped as described³⁵. The embryos were stained with an anti-PECAM antibody as described³⁹.

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Semaphorin 7A promotes axon outgrowth through integrins and MAPKs

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Striking parallels exist between immune and nervous system cellular signalling mechanisms. Molecules originally shown to be critical for immune responses also serve neuronal functions, and similarly neural guidance cues can modulate immune function. We show here that semaphorin 7A (Sema7A), a membrane-anchored member of the semaphorin family of guidance proteins previously known for its immunomodulatory effects, can also mediate neuronal functions. Unlike many other semaphorins, which act as repulsive guidance cues, Sema7A enhances central and peripheral axon growth and is required for proper axon tract formation during embryonic development. Unexpectedly, Sema7A enhancement of axon outgrowth requires integrin receptors and activation of MAPK signalling pathways. These findings define a previously unknown biological function for semaphorins, identify an unexpected role for integrins and integrin-dependent intracellular signalling in mediating semaphorin responses, and provide a framework for understanding and interfering with Sema7A function in both immune and nervous systems.

It is becoming increasingly clear that the nervous and immune systems display considerable overlap in their molecular repertoire. Molecules originally thought to be immune-cell-specific are now found also to perform critical neuronal functions, and vice versa^{1–4}. These include molecules involved in crosstalk between these seemingly disparate systems, as well as cues with parallel functions. Importantly, these molecular relationships may provide valuable insights into function and disease in both systems.

Semaphorins constitute a large family of soluble and membrane-bound proteins first identified owing to their prominent contribution to repulsive axon guidance during development^{5,6}. Although the roles played by semaphorins in the immune system are less well characterized, they support the possibility of a dual role for semaphorin family members in neural and immune functions^{7,8}. The membrane-associated GPI (glycosylphosphatidylinositol)-linked semaphorin Sema7A (CDw108) was originally identified in a search for vertebrate homologues of virally encoded semaphorins^{9,10}. Sema7A is expressed by a variety of lymphoid and myeloid cells^{9–14}, and also by several pre- and postnatal neuronal populations¹⁰. Like its viral counterpart SemaVB¹⁵, Sema7A affects immune-cell function *in vitro*, including chemotaxis and cytokine production¹⁴. In addition, Sema7A defines the John–Milton–Hagen human blood group on erythrocytes, which has been implicated in a clinically benign autoimmune disorder¹⁶. Thus far, no neuronal function has been attributed to Sema7A. Sema7A and the viral semaphorins SemaVA and SemaVB bind to the receptor plexinC1 (VESPR, CD232) *in vitro*^{15,17}. However, although plexinC1 is necessary for mediating the immunological effects of SemaVA¹⁵ and although plexin receptors have been implicated in many semaphorin-mediated neuronal events^{5,6}, the functional significance of the interaction between Sema7A and plexinC1 remains unknown. We find here that Sema7A has pronounced effects on axon outgrowth, and that this activity is dependent upon integrin receptor, but not plexinC1, function. Therefore, integrin-mediated semaphorin signalling may be a general mechanism used in the development and function of both the nervous and immune systems.

Sema7A promotes axon outgrowth

To examine whether Sema7A serves a dual role by affecting both neuronal and immunological functions, we first analysed Sema7A expression in the nervous system by *in situ* hybridization. Sema7A is expressed in a variety of neuronal cell types and in glial cells during neural development as well as in the intact¹⁰ and regenerating adult nervous system, revealing potential roles for this protein during development, plasticity and regeneration (Fig. 1a, c; data not shown).

To determine whether Sema7A, like other semaphorins, modulates axon guidance, we performed three-dimensional collagen matrix assays using 293-EBNA cells that secrete either a soluble, alkaline phosphatase (AP)-tagged, version of human Sema7A (AP-Sema7A)¹⁰ or AP as a control. In the presence of an AP-Sema7A protein gradient, axons extending from explants derived from olfactory epithelium, olfactory bulb, cortex and dorsal root ganglia were more numerous and longer, while vomeronasal and spinal cord motor axons remained unaffected (Fig. 1d; data not shown).

Asymmetrical neurite growth in collagen matrix cultures (longer axons in proximal versus distal quadrants) hinted at attractive effects of Sema7A on axons. To study whether Sema7A steers axons and/or promotes axon growth, we analysed the trajectories of individual OB axons emerging parallel to AP-Sema7A-secreting cell aggregates¹⁸ (Fig. 1e–h). Surprisingly, most of these fibres persisted on a linear course, showing no Sema7A-mediated chemotropic response (Fig. 1g). In contrast, Slit2, an OB axon repellent¹⁹, reoriented axons away from the aggregate (Fig. 1h).

Because *in vivo* both GPI-anchored and soluble forms of Sema7A may exist¹⁴, we next assessed the ability of full-length Sema7A to promote axon growth. OB explants grown on non-neuronal cell layers presenting soluble Sema7A or full-length, GPI-linked, Sema7A, displayed longer and more profuse axon outgrowth compared to control cell layers (Supplementary Fig. 1). This demonstrates that Sema7A can increase axon growth in both soluble and membrane-bound forms.

Sema7A is necessary for axon tract formation

The strong effect of Sema7A on OB axon growth suggests Sema7A may modulate the establishment of neuronal connectivity in the

central olfactory system. OB output neurons, that is, mitral and tufted cells, receive sensory inputs from the OE, processing this olfactory information before relaying it to the olfactory cortex²⁰. At embryonic day (E)14 to E15 in rats, mitral cell axons initiate the formation of a discrete, caudally extending fibre bundle called the lateral olfactory tract (LOT). Around E17, collateral branches are elaborated from these projections which then invade the olfactory cortex²¹. Recent studies reveal some of the molecular mechanisms that regulate LOT axon pathfinding and branching^{22–24}. However, factors controlling other aspects of LOT formation, including growth and positioning, remain unknown. Interestingly, it has been demonstrated that growth of the LOT relies on unidentified membrane-bound protein(s) presented by the presumptive LOT region and the olfactory cortex^{25,26}. It is thus intriguing that at E15 *Sema7A* is expressed by OB neurons and, specifically, along the presumptive LOT trajectory (Fig. 2a, b). At E19, *Sema7A* expression

persists in the OB and throughout the olfactory cortex (Fig. 2c, e; data not shown).

To evaluate whether *Sema7A* stimulates formation of the LOT in its native environment, we placed AP-*Sema7A*-secreting cell aggregates adjacent to the presumptive LOT region in organotypic cultures of whole telencephalic hemispheres from E14 rat^{23,25} (Fig. 2f, g). Consistent with our previous observations that *Sema7A* enhances OB axon length and number, the LOT diameter was significantly increased at the position just caudal to the OB where olfactory axons fasciculate to form the LOT (defined here as '0 μm ') in the presence of *Sema7A*. This effect was also evident caudally in closer proximity to the olfactory cortex ('800 μm '). Furthermore, in the presence of *Sema7A*, LOT axons extended further into the olfactory cortex (Fig. 2h–j). However, the position of the LOT in the cultured telencephalon was unaltered, *Sema7A* did not affect collateralization of LOT axons, and individual LOT fibres

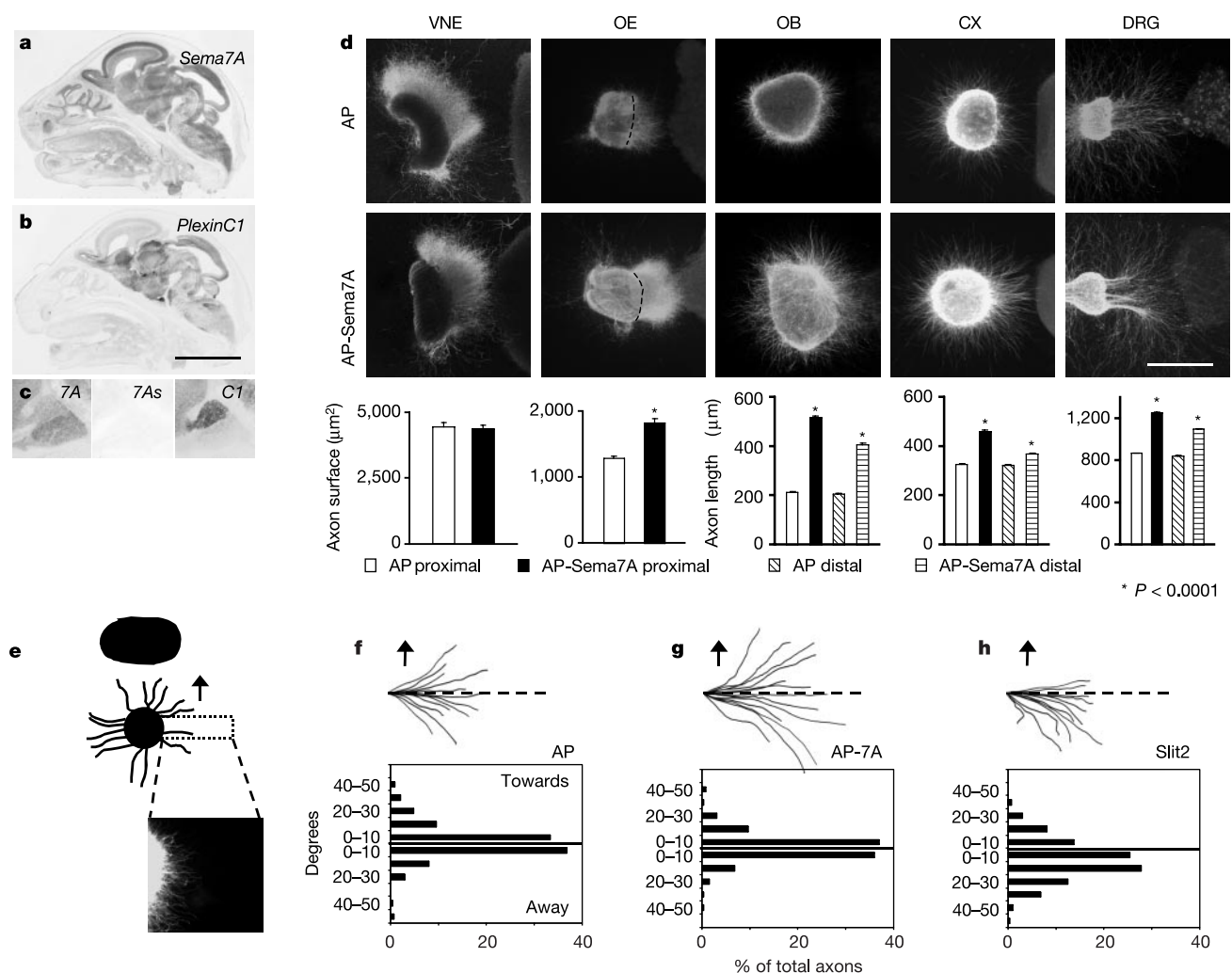


Figure 1 *Sema7A* promotes growth of central and peripheral axons. **a–c**, *In situ* hybridization⁴⁵ for *Sema7A* (**a**) and *plexinC1* (**b**) in adjacent sagittal sections through an E19 rat head. **c**, Higher magnifications of **a** and **b** show noticeable complementary expression in pituitary, whereas sense controls lack specific signals (7As; data not shown). **d**, E15 rat vomeronasal epithelium (VNE), olfactory epithelium (OE), olfactory bulb (OB), cortex (CX), and dorsal root ganglion (DRG) explants were cultured for 48–72 h in collagen matrix^{22,33} next to alkaline phosphatase (AP)-transfected 293-EBNA cells or stable 293-EBNA cells secreting AP-*Sema7A*¹⁰. Axons were labelled using antibodies to class III β -tubulin or neurofilament (2H3) and results were quantified by measuring total axon surface (VNE, OE) or by measuring the length of the 20 longest axons in the proximal

and distal quadrants (OB, CX, DRG)³⁰. Total number of explants quantified from four to five independent experiments ($n = \text{AP}, n = \text{AP-7A}$): VNE (20, 20), OE (24, 24), OB (39, 39), CX (22, 17), and DRG (14, 15). Asterisk, $P < 0.0001$ compared with AP. **e–h**, *Sema7A* does not induce directional growth of OB axons. **e**, E15 rat OB explants were cultured as in **d**. The trajectories of individual axons emerging parallel to the cell aggregates were analysed¹⁸. **f–h**, On top are camera lucida drawings of representative axons for each condition. Histograms depict how many degrees axons deviate from a straight course. For each experimental condition, 8–10 individual axons were randomly selected and analysed per explant ($n = 30$ explants each). Scale bars: **a, b**, 3,500 μm ; **c**, 1,080 μm ; **d**, 140 μm (VNE), 500 μm (OE, OB, CX), 1,000 μm (DRG); **e**, 390 μm ; **f–h**, 170 μm .

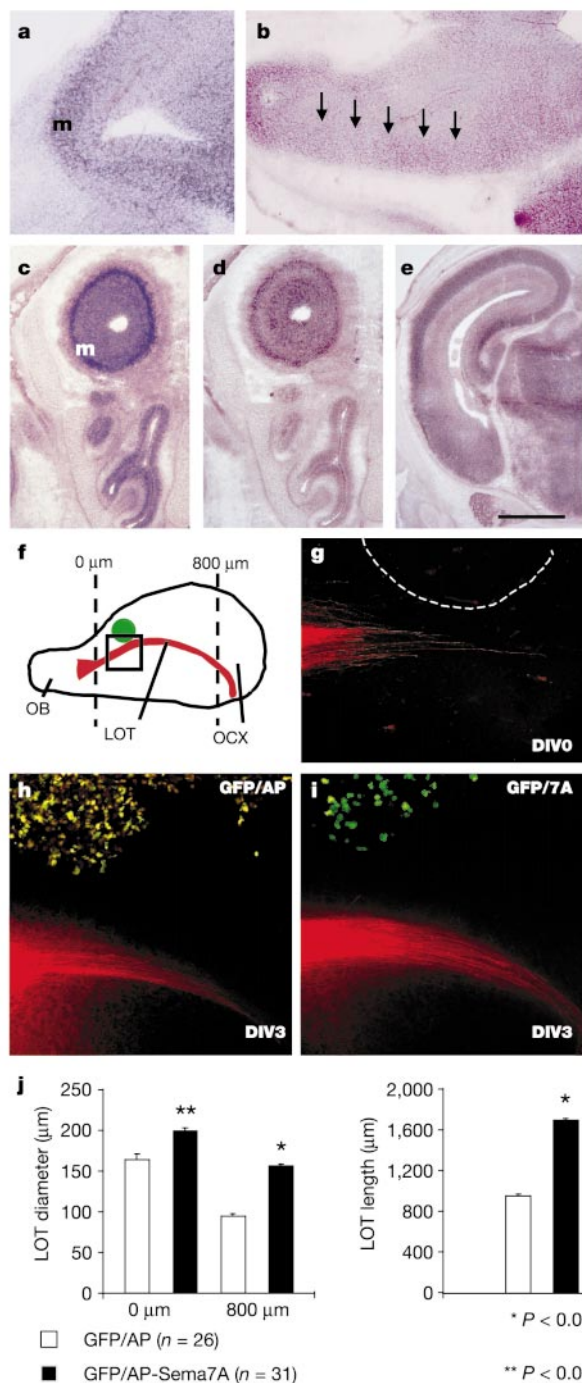


Figure 2 Sema7A stimulates lateral olfactory tract (LOT) formation. *Sema7A* (a–c, e) and *plexinC1* (d) messenger RNA expression in sagittal (a), horizontal (b), and coronal sections (c–e) through the E15 (a, b) and E19 (c–e) rat olfactory system. b, Arrows, *Sema7A* expression at the presumptive LOT position. m, mitral cells. Sense controls displayed no specific signals (not shown). f–j, *Sema7A* stimulates olfactory axon growth *in situ*. f, Green fluorescent protein (GFP)/AP (h) or GFP/AP-Sema7A (i) transfected cell aggregates were placed onto E14 rat whole-telencephalic cultures adjacent to the presumptive LOT position. After three days *in vitro* (DIV), the LOT was visualized by employing the lipophilic dye Dil (g–i)²³. g, Boxed area in f. Around E14, OB axons exit the olfactory bulb and start to fasciculate to form the LOT. Dashed line, presumptive position cell aggregate. h–j, To determine LOT extension the lengths of the five longest axons was measured starting at 0 μm. n, total number of cultures that were quantified from five experiments. OCX, olfactory cortex. Asterisk, $P < 0.0001$ and double asterisk, $P < 0.0002$ compared with GFP/AP. Scale bars: a, 300 μm; b, 750 μm; c, d, 980 μm; e, 1,150 μm; g–i, 300 μm.

displayed no tropic responses to Sema7A aggregates (Fig. 2h, i; data not shown).

We then explored the *in vivo* role of Sema7A during neural development by generating *Sema7A* null mice (Fig. 3a–e) and analysing development of the LOT in E16 wild-type, *Sema7A*^{+/−}, and *Sema7A*^{−/−} mice using retrograde Dil axon tracing²⁴ (Fig. 3f–n). *Sema7A* null mice were born at the expected mendelian frequencies from *Sema7A*^{+/−} intercrosses and appeared grossly normal. At E16 in mice, the LOT has completely formed and olfactory axon collaterals start to elaborate into the olfactory cortex²⁴ (Fig. 3f, g). In line with our gain-of-function experiment which indicated that Sema7A can stimulate growth of the LOT (Fig. 2f–j), mice lacking Sema7A showed impaired LOT outgrowth (Fig. 3f–j). In *Sema7A*-deficient mice the LOT, visualized in E16 whole-mount brains, was abnormally narrow compared to heterozygous and wild-type littermates, whereas tract positioning in the telencephalon was unaltered (Fig. 3f–j). It is unlikely that cell death in the OB contributes to the reduction in the LOT projection, because both OB cytoarchitecture and mitral/tufted cell numbers were unchanged in *Sema7A*-deficient mice (data not shown). Surprisingly, length of the LOT as visualized in E16 whole-mount brains was not significantly different in control and mutant mice (+/+ and +/−, 2,033 ± 30 μm ($n = 43$); −/−, 1,918 ± 35 μm ($n = 24$); $P > 0.05$). However, evaluation of Dil tracing in coronal sections through E16 brains, which allows for more-sensitive visualization of both LOT axons and collateral branches, showed that the overall LOT projection was substantially reduced in size in *Sema7A* mutants (Fig. 3k–n; $n = 5$ +/+, $n = 5$ +/−, and $n = 10$ −/−). Remarkably, in many *Sema7A* mutants examined using coronal sections no LOT was detectable at the most caudal levels, suggesting that in the absence of Sema7A the majority of OB axons fails to project into the most caudal parts of the olfactory cortex by E16 (Fig. 3n). Branching of the LOT appeared to be reduced in *Sema7A* mutants (Fig. 3f–n), however, this is probably due to overall impaired growth of the LOT since Sema7A does not affect OB axon branching *in vitro* or *in situ* (data not shown). The ability of a (reduced) LOT to form in *Sema7A*-deficient mice indicates that Sema7A acts combinatorially with other growth promoters to control the establishment of LOT projections. Although OB axons fail to respond to most of the neurotrophic factors²⁶, laminin and fibronectin enhance olfactory axon extension *in vitro* and may contribute to LOT growth *in vivo*²³. Overall, these experiments establish that Sema7A is an axon growth-promoting factor required for proper LOT development *in vivo*.

Sema7A-mediated axon growth is plexinC1-independent

We next performed AP-Sema7A binding experiments^{10,18} to visualize endogenous neuronal receptor(s) responsible for transducing Sema7A signals in olfactory neurons. In line with Sema7A effects on OB axons, AP-Sema7A avidly bound to cell bodies, axons and growth cones of dissociated OB neurons (Fig. 4a). Plexins and neuropilins have been shown to mediate semaphorin function in the nervous system⁵. Sema7A fails to bind neuropilin receptors^{10,17}. However, *in vitro* binding studies¹⁷, the strikingly complementary distribution of *Sema7A* and *plexinC1* in certain regions of the nervous system (Fig. 1a–c; data not shown), the strong *plexinC1* expression in the olfactory system (Fig. 2d) and the high levels of plexinC1 protein in dissociated OB neurons (data not shown) all support the possibility that plexinC1 serves as a neuronal Sema7A receptor. To test this hypothesis, we generated *plexinC1*-deficient mice and performed collagen matrix assays using tissues derived from mutant and wild-type mice. Remarkably, both AP-Sema7A binding to endogenous receptors and Sema7A-mediated axon growth of olfactory, cortical and sensory neurons were unaltered in the absence of plexinC1 (Fig. 4a–f; data not shown). By measuring OB axon length in the proximal quadrants of control and mutant cultures, we confirmed that wild-type and plexinC1-

To examine whether integrins mediate *Sema7A* function, we first altered the putative integrin-binding RGD sequence in *Sema7A* to KGE (Fig. 4g), which is not recognized by integrins³⁰, and tested the ability of substrates composed of the mutated protein 7A(KGE) to enhance axon growth. OB axon outgrowth on 7A(KGE) substrates was similar to AP control ($P > 0.05$) (Fig. 4j, p).

Next, we used synthetic RGD-containing peptides as competitive inhibitors of the *Sema7A* RGD binding site to interfere with integrin signalling²⁸ (Fig. 4g, q). Treatment of OB neurons grown on *Sema7A* substrates with RGD peptide³¹ dramatically reduced axon growth in a dose-dependent manner, whereas addition of a scrambled control peptide (P10)³⁰ had no effect (Fig. 4q; data not shown). We also assessed the ability of a 15-amino-acid RGD-containing peptide

derived from the *Sema7A* sequence to interfere with *Sema7A* signalling (7A-RGD; Fig. 4g). Unexpectedly, treatment with this 7A-RGD peptide led to a further increase in OB axon length, whereas this additive effect was completely abolished after a single amino acid substitution was made in the RGD sequence (7A-RAD: Fig. 4g, q). Furthermore, the 7A-RGD peptide, which is an amino acid sequence unique to *Sema7A*, promoted axon growth from neurons growing on control AP substrates, demonstrating a role for these *Sema7A* residues in enhancing axon growth (Fig. 4k, l, q). The agonistic effects of 7A-RGD were blocked by mutating the RGD sequence (that is, 7A-RAD), and by addition of RGD peptide (2.0 mg ml⁻¹), thus demonstrating that this effect is RGD-dependent (Fig. 4q). Taken together, these results strongly suggest

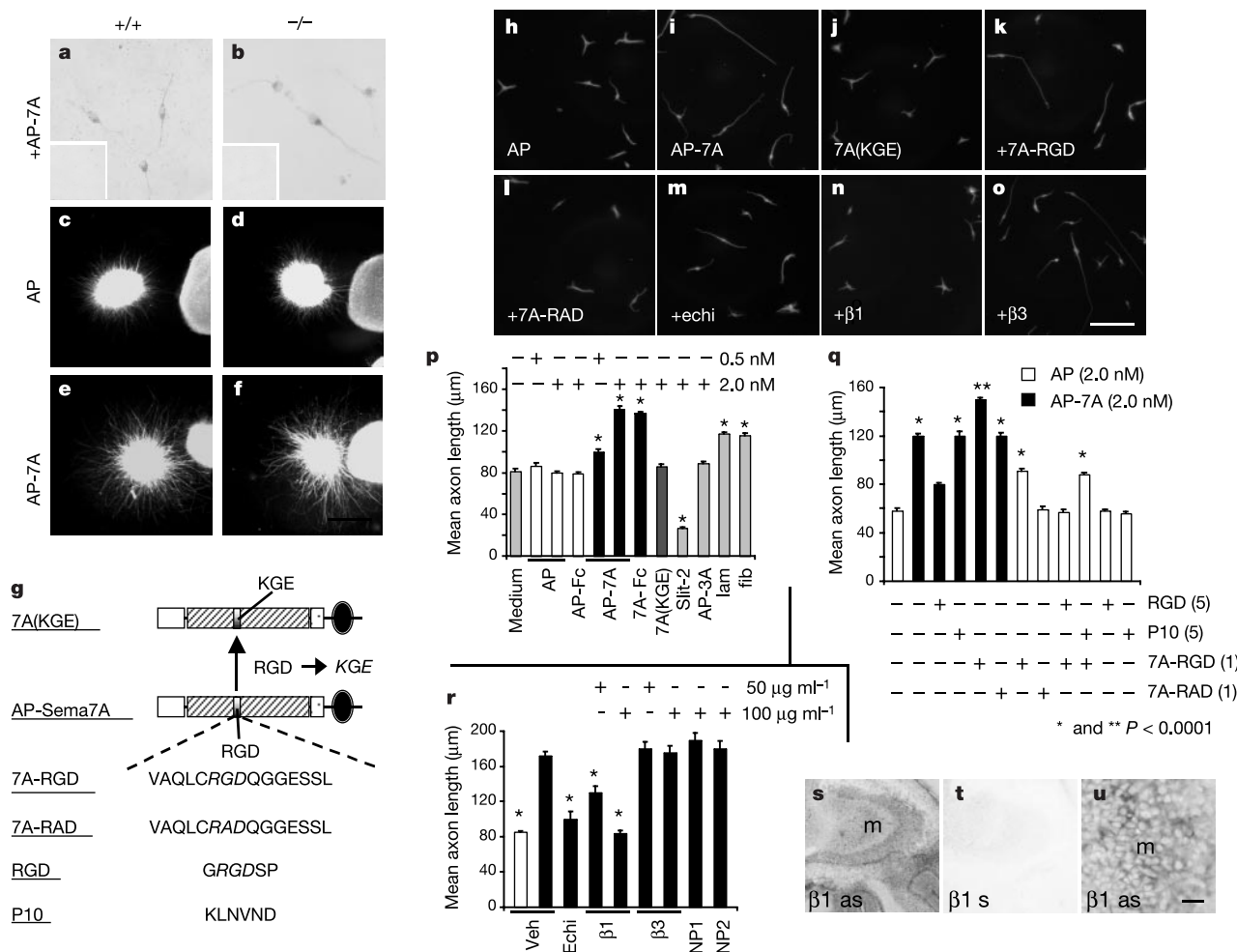


Figure 4 Integrins mediate *Sema7A* signalling. **a–f**, *Sema7A* enhances axon growth in a plexinC1-independent manner. **a, b**, AP-Sema7A binding¹⁰ to E14 dissociated OB neurons from wild-type (**a**) or *plexinC1*^{-/-} (**b**) mice. Insets show AP controls. **c–f**, Collagen matrix assays (see Fig. 1d) using E14 OB explants from wild-type (**c, e**) or *plexinC1*^{-/-} (**d, f**) mice. **g–u**, RGD-sensitive β1-subunit-containing integrins mediate axon growth promotion by *Sema7A*. **g**, Schematic drawings of soluble, AP-tagged (AP-Sema7A)¹⁰ and RGD-mutated, AP-tagged (7A(KGE)) *Sema7A* constructs, and synthetic peptides used in competition studies. **h–r**, Site-directed mutagenesis of the *Sema7A* RGD sequence, RGD peptides, and integrin function-blocking antibodies neutralize *Sema7A* axon growth. Dissociated neurons derived from E15 rat OB were cultured for 2–3 days on substrate-coated coverslips²³, fixed and processed for immunocytochemistry with anti-β tubulin or anti-tau (not shown) antibodies. **h–o**, Representative examples of neurons cultured on AP- (**h, k, l**), AP-Sema7A- (**i, m–o**)

or 7A-KGE substrates (**j**) in the absence (**h–j**) or presence of peptides (**k, l**), inhibitor (**m**), or antibodies (**n, o**). **p–r**, Axon-length quantification³⁰ of neurons cultured on various substrates (**p**), on AP- or AP-Sema7A substrates in the presence of different synthetic peptides or combinations thereof (peptide concentrations in mg ml⁻¹ are in parentheses) (**q**), or on AP- and AP-Sema7A substrates and treated with the β1/β3-specific integrin inhibitor (echistatin (Echi); 1.0 μM) or function-blocking antibodies to β1, β3, neuropilin (NP)-1 or NP-2 (**r**). Final concentration of fusion protein substrates is 0.5 or 2.0 nM. Veh, vehicle. Data represent means ± s.e.m. of at least four independent experiments. Asterisk, $P < 0.0001$ compared with AP (**p, q**) or AP-7A (**r**). Double asterisk, $P < 0.0001$ compared with AP-7A. **s, u**, β1 expression in the E18 rat olfactory system. **t**, Sense control. fib, fibronectin; lam, laminin; m, mitral cell. Scale bars: **a, b**, 50 μm; **c–f**, 300 μm; **h–o**, 170 μm; **s, t**, 290 μm; **u**, 70 μm.

that an RGD-sensitive integrin is essential for mediating the axon growth-promoting responses of *Sema7A*.

Integrins are functional heterodimers composed of α and β subunits. There are 18 α and 8 β subunits, and they associate to form 24 different integrin receptors with differing ligand specificities²⁷. Several α and β subunits expressed in the nervous system can form RGD-recognizing integrins^{27,28}. The disintegrin echistatin, a viper-venom-derived RGD peptide that specifically inhibits $\beta 1$ - and $\beta 3$ -containing integrins³², dramatically reduced *Sema7A*'s effect on OB axon growth (Fig. 4m, r). To discriminate between $\beta 1$ - and $\beta 3$ -containing integrins, we used function-blocking antibodies which completely inhibit the activity of these subunits³¹. The blockade of $\beta 1$ -containing receptors with anti- $\beta 1$ monoclonal

antibodies (mAbs) neutralized axon growth promotion by *Sema7A* in a dose-dependent manner (Fig. 4n, r). In contrast, anti- $\beta 3$ mAbs and anti-neuropilin-1 and -2 function-blocking antibodies³³ had no effect ($P > 0.05$) (Fig. 4o, r). Consistent with these results, robust levels of $\beta 1$ transcripts are observed in *Sema7A*-responsive neurons including mitral cells (Fig. 4s–u; data not shown). Thus, a $\beta 1$ -subunit-containing integrin receptor mediates the growth-promoting effects of *Sema7A* on axons.

Sema7A activates FAK and MAPKs

Ligand-induced integrin clustering leads to the activation and recruitment of intracellular signalling and structural proteins to focal adhesion sites²⁷. The non-receptor protein kinase focal adhesion kinase (FAK) plays a central role in $\beta 1$ integrin-dependent signalling. FAK is rapidly phosphorylated upon ligand binding, leading to the activation of cytoskeleton-linked proteins responsible for signal propagation downstream of integrins. To determine whether *Sema7A* elicits integrin-dependent signalling responses, we treated OB neurons with AP- or AP-*Sema7A*-conditioned medium and assessed tyrosine phosphorylation of FAK. AP-*Sema7A* increased FAK phosphorylation in neurons, confirming a role for integrin-associated signal transduction machinery in propagating *Sema7A* signals (Fig. 5a).

An important function of FAK is the regulation of mitogen-activated protein (MAP) kinase pathways³⁴. MAPKs have been implicated in neurite growth stimulated by cell adhesion molecules and neurotrophic factors^{35,36}. Interestingly, MAPK signalling also underlies neurite outgrowth of PC12 cells by the secreted semaphorin *Sema3E*³⁷ and the potentiation of neurotrophin-mediated PC12 neurite outgrowth by the transmembrane semaphorin *Sema4D*³⁸. To investigate whether MAPK cascades are involved in neuronal *Sema7A* signalling, dissociated OB neurons were stimulated with AP- or AP-*Sema7A*-conditioned medium, after which phosphorylation of the MAPKs extracellular regulated kinases (ERK) 1 and 2 was assessed. *Sema7A* caused a rapid and sustained increase in the phosphorylation of ERK1/2, whereas AP had no effect (Fig. 5b, Supplementary Fig. 3). In contrast, treatment with 7A(KGE) failed to trigger ERK1/2 phosphorylation, showing that the integrity of the RGD motif, and thus interactions with integrins, are essential for activation of MAPK signalling by *Sema7A* (Fig. 5b). To determine whether ERK activation is required for *Sema7A* function, we cultured neurons in the presence of the U0126, a specific inhibitor of MAPK kinases (MEK)-1 and MEK-2 that blocks ERK1/2 phosphorylation. U0126 blocked *Sema7A*-mediated axon growth stimulation in a dose-dependent manner, whereas the related but inactive compound U0124 had no effect (Fig. 5c). Therefore, MAPK-dependent signalling mechanisms are required for axon outgrowth stimulation by *Sema7A*.

Discussion

These studies describe a new biological function for semaphorins, establish an unexpected role for integrins as mediators of semaphorin responses, and provide insight into the molecular basis of semaphorin-dependent intracellular signalling. Mutation of the *Sema7A* integrin-binding motif and RGD blocking peptides, both commonly used methods to abrogate interactions between integrins and their ligands^{28,30–32}, blocks *Sema7A*-mediated axon growth. This suggests that *Sema7A* signalling in neurons may involve direct interactions of *Sema7A* and a $\beta 1$ -integrin(s). However, the requirement for $\beta 1$ -integrins in mediating *Sema7A*-induced axon growth does not exclude the possibility that other molecules are involved in mediating *Sema7A* functions. Integrins are known to associate with a large number of distinct proteins²⁷. Thus, $\beta 1$ -integrins may constitute obligate co-receptors which associate with unidentified *Sema7A*-binding proteins. All neural semaphorin signalling has been assumed to be mediated by multimeric receptor complexes containing plexins as obligatory signal transducing subunits⁵.

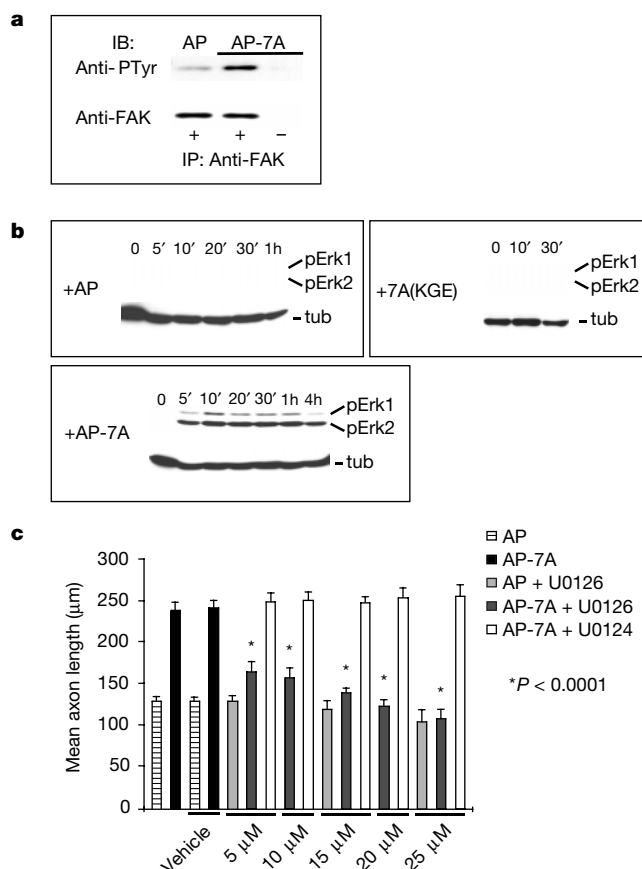


Figure 5 Integrin-induced MAPK signalling is necessary for *Sema7A*-mediated axon growth. **a**, *Sema7A* increases phosphorylation of FAK. E15 dissociated OB neurons were grown on 0.02% poly-L-lysine for 16 h, treated with AP- or AP-*Sema7A*-conditioned medium (20 nM) for 30 min, and subjected to immunoprecipitation (IP) using anti-FAK antibodies (+) or rabbit IgG (–)⁴⁶. The samples were immunoblotted with anti-phosphotyrosine antibodies. After stripping, the blots were reprobed with anti-FAK to control the levels of IP-ed FAK⁴⁶. Experiments were done three times with similar results. **b**, *Sema7A* induces ERK1/2 phosphorylation in an RGD-dependent manner. E15 dissociated OB neurons were treated with AP-, AP-*Sema7A*- or 7A(KGE)-conditioned medium (20 nM) for 5 min to 4 h as described above, and subjected to immunoblotting with an antibody specific for the phosphorylated forms of ERK1 and ERK2. The immunoblot was later stripped and reprobed for α -tubulin to normalize for protein amounts²⁹. Experiments were done three times with similar results. **c**, The MEK inhibitor U0126 neutralizes axon growth responses by *Sema7A*. Dissociated OB neurons grown on AP- or AP-*Sema7A* substrates (2.0 nM) were untreated or treated with vehicle (0.1% DMSO), U0126 (5–25 μ M), or negative control U0124 (5–25 μ M). Quantification of axon length was determined as described³⁰ from three independent experiments. Asterisk, $P < 0.0001$ compared with AP-7A (untreated, vehicle or U0124).

However, *Sema7A*-mediated axon growth is likely to be plexin-independent since plexinC1 is not required for this effect and *Sema7A* fails to bind vertebrate plexins other than plexinC1¹⁷ (Z. He and L. Tamagnone, personal communications; data not shown). Intriguingly, semaphorin proteins belonging to different classes possess evolutionarily conserved integrin-binding motifs (*Sema3C*, *Sema4G*) suggesting that additional semaphorin-integrin interactions may occur. Furthermore, several other guidance cues with neurite-outgrowth-promoting activities contain integrin-binding motifs (including netrin-1 and certain class A ephrins)^{39,40}. Therefore, integrin-mediated signalling mechanisms may be critical in mediating positive effects of axon guidance cues on axons.

Sema7A is expressed by several different classes of lymphoid and myeloid cells and is a potent immunomodulator^{13,14}. Host-lymphocyte *Sema7A* is incorporated in the HIV-1 envelope during the budding stage, and it is suspected to be involved in HIV-1 infection⁴¹. *Sema7A* is also present in microglia, the principal immune effector cells of the nervous system, which are postulated to arise from blood monocytes¹⁴. Since *Sema7A* is an autocrine monocyte activator¹⁴ and *Sema7A* expression is upregulated following spinal cord injury (data not shown), *Sema7A* may participate in post-injury neural immune responses. The discrepancy between the biological activity of *Sema7A* during monocyte activation (0.1–1 pM) (ref. 14) and the affinity of *Sema7A* for plexinC1 ($K_D = 2.1$ nM)¹⁷ suggests plexinC1 might not mediate all aspects of immunological *Sema7A* function. Given their role as mediators of neuronal *Sema7A* functions, integrins seem likely candidates to mediate the immunomodulatory effects of *Sema7A*. This is supported by the fact that integrins control several aspects of immune function⁴², the observation that *Sema7A* induces monocyte aggregation, suggesting an interaction with cell surface adhesion proteins such as integrins¹⁴, and expression of integrin receptors by monocytes and microglia^{42,43}. Thus, *Sema7A* and the signalling machinery it activates may constitute targets for intervention in pathological immune responses and neural regeneration. □

Methods

Reagents and antibodies

Blocking peptides (JHU, HHMI Biopolymer lab); Echistatin, anti- α - or β -tubulin mAbs (Sigma); laminin, fibronectin (40 μ g ml⁻¹; Gibco); U0126, U0124, anti-pERK (Cell Signaling); DII, Hoechst 33258 dye (Molecular Probes); hamster anti-rat β 1 mAb (Clone Ha2/5), mouse anti-rat β 3 mAb (Clone F11, Pharmingen); anti-neurofilament mAb (2H3; Developmental Studies Hybridoma Bank); rat anti-mouse plexinC1 mAb (m653; Amgen); anti-tau mAb (Chemicon); rabbit anti-FAK (A-17; Santa Cruz); anti-phosphotyrosine (4G10; Upstate Biotechnology).

Cell and tissue culture

293-EBNA cells were cultured and transfected as described^{10,33}. Inhibitors, blocking antibodies and peptides were added to the culture medium which was refreshed each day. Conditioned media were prepared as described²³ and concentrations of fusion proteins were determined by western blotting and soluble AP assays. Substrate-coated coverslips were prepared with recombinant proteins or conditioned media from transiently or stably transfected 293 EBNA cells^{23,30}. Routinely, a 10% adsorption of input protein to poly-L-lysine-coated coverslips was observed³⁰ (data not shown). Therefore, 5 and 20 nM of input protein correspond to 0.5 and 2.0 nM final concentrations (Figs 4 and 5). Neuronal survival was assessed by employing Hoechst 33258 chromatin stains²⁹. All axon and tract measurements and cell counts were performed using a CCD camera and Openlab software. Data were plotted as a mean $\pm$ s.e.m. and analysed with ANOVA or paired *t*-tests.

Site-directed mutagenesis and plasmid constructs

The four-primer method³⁰ was employed to mutate the RGD sequence of AP-*Sema7A* to KGE using the following primers: 5'-CGGGATCCACCTAAGGAGCGGACCCCGC-3', 5'-CCCTCGAGCAGCAGGTGCTCGGCCATG-3', 5'-CAGGGTGGGGAAAGTTCACTGTCAGTC-3', and 5'-CCCCACCCTGCTCCCCCTTGAC-3' (mutations are in italics).

The following DNA constructs were used: pEGFP-N1 (Clontech); pAP-TAG4 (GenHunter); pCIneo-plexinC1, which encodes full-length mouse plexinC1; pB-rSema7A, which encodes base pairs 364–726 of rat *Sema7A*; pAP-Sema3A, pEX-AP-Sema7A and pEX-Sema7A-Fc have been described^{10,33}; pBSK- β_{OL} encoding rat β 1 integrin, pSECTag-hSlit2, encoding myc-tagged full-length human Slit2, pCMV-CDw108, encoding mouse *Sema7A*, and pCDNA-AP-Fc/myc were provided by L. H. Rome, M. Tessier-Lavigne, A. Yamada and R. J. Giger, respectively.

Generation of *Sema7A* and *plexinC1* deficient mice

A BAC containing a 5' portion of the *Sema7A* gene was obtained from Incyte Genomics. BAC DNA fragments and a *neo FRT/loxP* cassette (provided by K. Takamiya and R. Huganir, Johns Hopkins School of Medicine) were used to generate a conditional *Sema7A* targeting vector using standard recombinant DNA techniques (Fig. 3a). Following electroporation of the *Sema7A*-based targeting vector, targeted 129-derived ES clones were identified and injected into C57BL/6 blastocysts. Resulting male chimaeras were bred to C57BL/6 females to generate heterozygous mice carrying the mutation. *Sema7A* null mice were obtained by crossing F₁ heterozygous mice with mice that express Cre recombinase in the germ-cell lineage⁴⁴. The resulting *Sema7A* mutation was then moved into a C57BL/6 background by three successive backcrosses. PCR genotyping was performed using the following primers (Fig. 3a); P1, 5'-GGCCACAGGATTCAGTGCA GGCC-3'; P2, 5'-CCACAGACCCAGACATACTAGAGC-3'; P3, 5'-CCCGCTGCCAGC AGAGCTCGC-3'.

For details on the generation of *plexinC1* null mice see Supplementary Fig. 2.

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Molecular gas in the host galaxy of a quasar at redshift $z = 6.42$

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Observations of molecular hydrogen in quasar host galaxies at high redshifts provide fundamental constraints on galaxy evolution, because it is out of this molecular gas that stars form. Molecular hydrogen is traced by emission from the carbon monoxide molecule, CO; cold H_2 itself is generally not observable. Carbon monoxide has been detected in about ten quasar host galaxies with redshifts $z > 2$; the record-holder is at $z = 4.69$ (refs 1–3). Here we report CO emission from the quasar SDSS J114816.64 + 525150.3 (refs 5, 6) at $z = 6.42$. At that redshift, the Universe was only 1/16 of its present age, and the era of cosmic reionization was just ending. The presence of about $2 \times 10^{10} M_\odot$ of H_2 in an object at this time demonstrates that molecular gas enriched with heavy elements can be generated rapidly in the youngest galaxies.

The source, which we refer to as J1148 + 5251 in this Letter, is a luminous quasar which is thought to be powered by mass accretion onto a supermassive black hole of mass $(1\text{--}5) \times 10^9 M_\odot$ where the subscript $\odot$ refers to the Sun⁷. Optical spectra of J1148 + 5251 show a clear Gunn–Peterson trough^{5,6} (that is, Ly α absorption by the neutral intergalactic medium, IGM), indicating that this quasar is situated at the end of the epoch of reionization⁸. Thermal emission from warm dust was detected at millimetre wavelengths, implying a far-infrared (FIR) luminosity of $1.3 \times 10^{13} L_\odot$ (ref. 9), corresponding to about 10% of the bolometric luminosity of the system (we assume $H_0 = 71 \text{ km s}^{-1} \text{ Mpc}^{-1}$, $\Omega_\Lambda = 0.73$ and $\Omega_m = 0.27$ throughout). We searched for molecular gas in J1148 + 5251 using the National Radio Astronomy Observatory's (NRAO) Very Large Array (VLA) and the IRAM Plateau de Bure interferometer (PdBI) in February–April 2003. The VLA observations have higher spatial resolution, whereas the PdBI has better spectral resolution. We observed the CO(3–2), (6–5) and (7–6) lines which are shifted to 46.6, 93.2 and 108.7 GHz, respectively, at $z = 6.42$. For J1148 + 5251 the possible redshift range based on broad optical emission lines is $z = 6.35$ (ref. 5) to 6.43 (ref. 7). We searched this entire range for CO(3–2) emission using the VLA at a resolution of 50 MHz (320 km s^{-1} , $\Delta z = 0.008$) per channel. CO(3–2) emission is clearly detected at high signal-to-noise ratio in the channel centred at 46.6149 GHz (see Figs 1 and 2), corresponding to a redshift of $z = 6.418 \pm 0.004$. The PdBI covered a redshift range from $z = 6.40\text{--}6.44$ and the CO emission is also detected at high significance (Fig. 2)¹⁰. Here we concentrate on the VLA results; a more detailed analysis of the entire data set will be presented elsewhere¹⁰.

The velocity-integrated CO(3–2) flux is $S_{\text{CO}(3-2)}\Delta\nu = 0.18 \pm 0.04 \text{ Jy km s}^{-1}$. On the Kelvin scale (1 Jy = 250 K for a $1.5''$ beam) the source has a peak line flux of 0.14 K and a line integral of $44 \pm 10 \text{ K km s}^{-1}$. The CO luminosity L' (in $\text{K km s}^{-1} \text{ pc}^2$) is

given by¹¹:

$$L' = 3.25 \times 10^7 \times S_{\text{CO}} \Delta\nu \times \nu_{\text{obs}}^{-2} \times D_L^2 \times (1+z)^{-3}$$

where D_L is the luminosity distance in Mpc and ν_{obs} is given in GHz. For J1148 + 5251 we obtain: $L'_{\text{CO}(3-2)} = 2.7 \times 10^{10} \text{ K km s}^{-1} \text{ pc}^2$. The total molecular gas mass, $M(H_2)$, can be derived from the relation $M(H_2) = \alpha \times L'_{\text{CO}(1-0)}$. In Galactic molecular clouds the conversion factor¹² is $\alpha \approx 2.0$ (in units of $M_\odot (\text{K km s}^{-1} \text{ pc}^2)^{-1}$), but here we use the value $\alpha \approx 0.8$ (in units of $M_\odot (\text{K km s}^{-1} \text{ pc}^2)^{-1}$), appropriate for ultraluminous infrared galaxies¹³ ($L_{\text{FIR}} \geq 10^{12} L_\odot$) and nuclear starburst galaxies¹⁴. Assuming a constant brightness temperature from CO(3–2) to CO(1–0)¹⁰, this leads to $M(H_2) = 2.2 \times 10^{10} M_\odot$ for J1148 + 5251.

If the FIR luminosity of J1148 + 5251 is powered by star formation, the implied star formation rate is $3,000 M_\odot$ per year (ref. 9). Comparing this to the molecular gas mass implies a short gas depletion timescale of the order of 10^7 years. However, given the high luminosity of the quasar it is possible that some fraction of the dust is heated by the quasar, in which case the star formation rate quoted above becomes an upper limit (resulting in a longer gas depletion timescale). The FIR continuum-to-CO line ratio for J1148 + 5251 is large, 440 (in $L_\odot (\text{K km s}^{-1} \text{ pc}^2)^{-1}$), which is an order of magnitude larger than for normal star-forming galaxies¹⁵ (5–50), but is comparable to that seen in ULIRGs and other high redshift quasars⁹. Some authors have suggested that high continuum-to-line ratios indicate high star-formation efficiencies¹⁵. Alternatively, dust heating by the active galactic nuclei (AGN) could lead to large continuum-to-line ratios.

A dynamical mass for the system can be derived from the CO observations, assuming that the CO is rotationally supported. The upper limit to the CO source diameter derived from gaussian fitting is $1.5''$ ($1'' = 5.46 \text{ kpc}$). A lower limit to the source diameter can be derived from the measured brightness temperature (T_{obs}) by assuming an intrinsic brightness temperature T_b : $\Omega_s/\Omega_B = (T_{\text{obs}}/T_b)(1+z)$, where Ω_s and Ω_B are the source and beam solid angles. The most extreme case is a single optically thick emission region, in which case T_b equals $T_{\text{ex}} - T_{\text{CMB}}$, the excitation brightness temperature minus the continuum temperature of the cosmic microwave background at $z = 6.4$ ($\sim 20 \text{ K}$). This gives a minimum source diameter of $0.2'' (T_b)^{-1/2}$ where T_b is in units of 50 K. Assuming a rotational velocity of $\nu_{\text{rot}} = 130 \sin^{-1}(i)$ (in units of km s^{-1} (Fig. 2), where i is the inclination angle with respect to the sky plane, yields a range for the dynamical mass of $M_{\text{dyn}} = (2\text{--}16) \times 10^9 \times \sin^{-2}(i) M_\odot$. This is comparable to the molecular gas mass in J1148 + 5251, implying that either molecular gas dominates the dynamics of the system, or that the plane of rotation is close to the sky plane. The optical spectrum of the quasar shows no significant reddening⁵, despite the large dust mass of the host galaxy⁹. This suggests that the disk of molecular gas (and dust) is oriented close to the sky plane such that the line of sight to the nucleus is relatively unobscured. We note that the mass of the central black hole⁴ probably constitutes a significant fraction ($\sim 1\text{--}10\%$) of the total dynamical mass in the inner few kiloparsecs of J1148 + 5251, in contrast to what is generally found for nearby galaxies^{16,17}.

The CO redshift of $z = 6.419$ (Fig. 2) corresponds to the systemic redshift of the host galaxy of J1148 + 5251, since it traces the extended molecular gas distribution in the quasar and is not associated with emission supposedly emerging from energetic processes, such as lines of shocked outflow gas or gas related to AGN accretion. The latter has been found to be shifted significantly in frequency with respect to the systemic redshift¹⁸. Indeed, studies of high ionization ultraviolet lines (in particular SiIV)⁵ in J1148 + 5251 yield a redshift of $z = 6.37$ corresponding to a velocity offset of $\sim 2,000 \text{ km s}^{-1}$. The CO redshift is however in good agreement with the redshift derived from the low ionization MgII line⁷.

Measuring an accurate redshift is particularly important to

determine the state of the IGM around the quasar (the 'proximity effect'). In the optical spectrum^{5,7} there is essentially zero emission present in the wavelength range corresponding to Ly α at $z = 5.7$ – 6.33 (due to the Gunn–Peterson effect); emission for $z > 6.33$ can

be attributed to the ionized medium around the quasar. Using the source redshift of 6.419 results in a Strömberg sphere around J1148 + 5251 with a comoving radius of $R_S = 4.8$ Mpc. An estimate for the age for this sphere (and hence for the quasar itself) can be derived from R_S using^{19,6}

$$\dot{N}_{\text{ph}} \times t_q = 0.34 \times [R_S \times (1 + z_q) / 7.419]^3,$$

where $\dot{N}_{\text{ph}}$ is in units of 10^{58} s^{-1} , t_q in units of 10^7 yr , R_S in units of 4.8 Mpc, and assuming $\dot{N}_{\text{ph}} = (0.2\text{--}1.3) \times 10^{58} \text{ s}^{-1}$ (refs 6, 20, 21). This calculation results in an age of order 10^7 yr for the quasar activity in J1148 + 5251. Interestingly, this timescale is comparable to the formation timescale for the central black hole, which has an e -folding timescale for accretion of order $4 \times 10^7 \text{ yr}$ (assuming a radiative efficiency of 0.1 and that the black hole is accreting at the Eddington limit)⁷, suggesting that the AGN ionizes a significant volume around the quasar.

The fact that we detect CO emission implies that the process of enrichment of the ISM in J1148 + 5251 with heavy elements is relatively advanced, that is, that significant star formation has occurred in the quasar host galaxy prior to $z = 6.4$. This conclusion is supported by the fact that both strong metal emission lines⁵, and thermal emission from warm dust⁹, were detected from J1148 + 5251. A recent optical study of a $z = 6.28$ quasar even suggests supersolar metallicities in these early objects²². Assuming a Galactic abundance for J1148 + 5251 we derive an order-of-magnitude estimate for the total mass in CO of $M(\text{CO}) \approx 3 \times 10^7 M_\odot$ (ref. 10). This amount of C and O could be produced relatively quickly by $\sim 10^7$ hundred solar-mass population III stars²³ with lifetimes $< 10^7 \text{ yr}$. Likewise, if enrichment was achieved by conventional supernovae²⁴ and assuming a star-formation rate of order $3,000 M_\odot \text{ yr}^{-1}$ (ref. 9), we estimate a time for enrichment of order 10^7 yr . We consider this time a lower limit for enrichment of the ISM since redistribution and cooling of the gas will occur on timescales of order 10^8 yr . This implies that the ISM enrichment process in J1148 + 5251 presumably started at redshifts $z > 8$. This is in

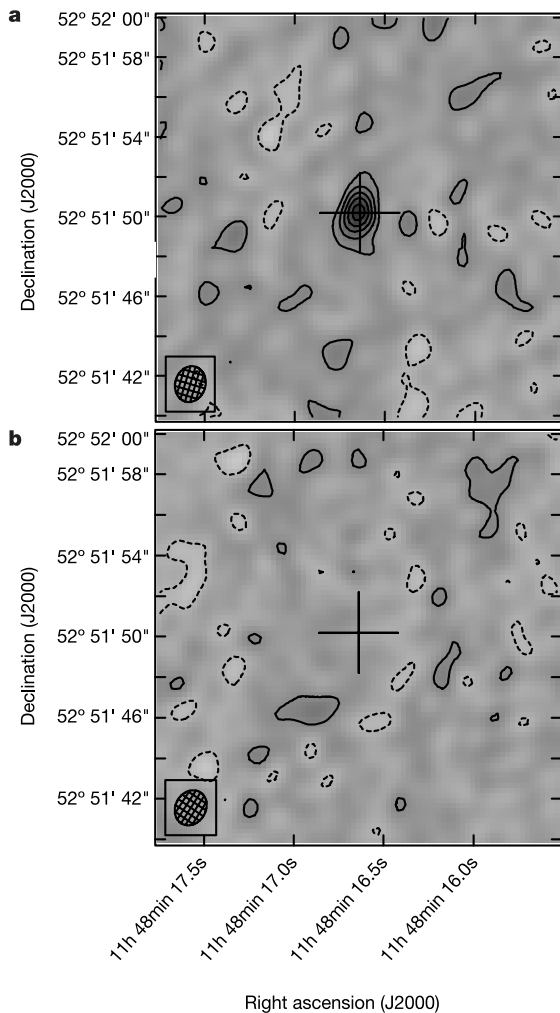


Figure 1 CO detection in J1148+5251. **a**, VLA CO(3-2) emission detected at 46.6149 GHz ($z = 6.418$, bandwidth: 50 MHz, $\Delta z = 0.008$). **b**, Co-added VLA emission in the neighbouring line-free channels. The (1σ) noise in both images is $0.057 \text{ mJy beam}^{-1}$ and contours are plotted at $-0.1, 0.1, 0.2, 0.3, 0.4$ and 0.5 mJy per beam . The peak flux in **a** is $0.570 \text{ mJy per beam}$; that is, J1148+5251 is detected at 10σ . On the basis of the line-free channels we derive a 2σ upper limit to the continuum emission at 46.6 GHz (restframe wavelength = $870 \mu\text{m}$) of 0.11 mJy . The cross indicates the optical position of J1148+5251. The optical and radio positions are coincident within the uncertainties ($\sim 0.1'' = 0.6 \text{ kpc}$). The observations were taken with the VLA in the most compact configuration (D array, maximum baseline: $\sim 1 \text{ km}$, leading to a resolution of $1.8'' \times 1.5''$; the beam is plotted in the lower left of each panel). Only two 50-MHz channels can be observed at once with the VLA, so the source has been observed repeatedly with different frequency settings; observations were made using two polarizations and 2 IFs of 50 MHz each, scanning in frequency from 47.065 GHz to 46.515 GHz, corresponding to a CO(3-2) redshift range of 6.347 to 6.434. The observing time for the channels shown are $\sim 20 \text{ h}$, respectively. Observations were obtained in fast switching mode, and the phase stability was excellent at all times. We used the nearby quasar 11534+49311 (flux density = 1.6 Jy at 46.6 GHz) for phase and amplitude calibration. The absolute flux calibration was derived by observing 3C286. No evidence for strong gravitational lensing is seen in optical images⁵, rendering strong magnification unlikely. However, the presence of an intervening galaxy at $z = 4.9$ has been suggested, based on optical spectroscopy⁶, and counts of radio and optical sources in the vicinity of J1148+5251 argue for a foreground cluster, such that weak magnification (about a factor of two) is possible^{5,9}.

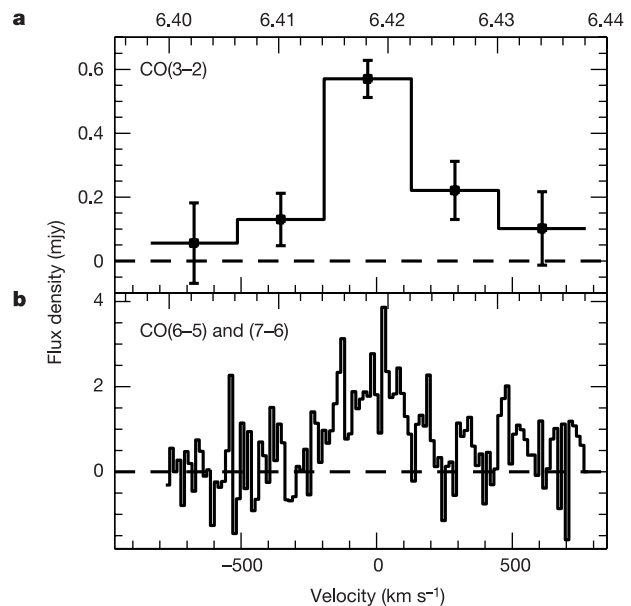


Figure 2 The CO spectrum of J1148+5251. **a**, The spectrum of the CO(3-2) line of J1148+5251 at 320 km s^{-1} resolution as observed with the VLA. The plotted errors are $\pm 1\sigma$. **b**, The averaged CO(6-5) and CO(7-6) observations from the PdBI¹⁰ (channel width = $5 \text{ MHz} = 13.8 \text{ km s}^{-1}$, noise per channel = 0.8 mJy , beam size $\approx 5''$) to demonstrate the consistency of the results obtained by the two instruments. A gaussian fit to the PdBI data gives a velocity width of 250 km s^{-1} and a redshift of $z = 6.419$ (corresponding to '0' velocity)¹⁰.

agreement with the recent results from the Wilkinson Microwave Anisotropy Probe (WMAP) which indicate that the first onset of star formation most probably occurred at redshifts $z \approx 15\text{--}20$ (~ 250 Myr after the Big Bang)^{25–27}.

The presence of Ly α emitting galaxies and luminous quasars at the end of cosmic reionization ($z > 6.0$), at a time when the IGM was at least 1% neutral, was clearly demonstrated^{5,28}. This epoch of reionization represents a key benchmark in cosmic structure formation, indicating the formation of the first luminous structures. Detecting a large reservoir of molecular gas in this epoch demonstrates the existence of the requisite fuel for active star formation in primeval galaxies. The existence of such reservoirs of molecular gas at early times implies that studies of the youngest galaxies will be possible at millimetre and centimetre wavelengths, unhindered by obscuration by the neutral IGM. □

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Rotational actuators based on carbon nanotubes

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Nanostructures are of great interest not only for their basic scientific richness, but also because they have the potential to revolutionize critical technologies. The miniaturization of electronic devices over the past century has profoundly affected human communication, computation, manufacturing and transportation systems. True molecular-scale electronic devices are now emerging that set the stage for future integrated nanoelectronics¹. Recently, there have been dramatic parallel advances in the miniaturization of mechanical and electromechanical devices². Commercial microelectromechanical systems now reach the submillimetre to micrometre size scale, and there is intense interest in the creation of next-generation synthetic nanometre-scale electromechanical systems^{3,4}. We report on the construction and successful operation of a fully synthetic nanoscale electromechanical actuator incorporating a rotatable metal plate, with a multi-walled carbon nanotube serving as the key motion-enabling element.

The overall size scale of our actuator is of the order of ~ 300 nm and its components are integrated on a silicon chip. Low-level externally applied voltages precisely control the operation speed and position of the rotor plate. Repeated oscillations of the rotor plate between positions 180° apart, as well as rotations of 360° , have been demonstrated with no signs of wear or fatigue. Unlike existing chemically driven bio-actuators and bio-motors, our fully synthetic nanometre-scale electromechanical system (NEMS) actuator is designed to operate over a wide range of frequency, temperature, and environmental conditions, including high vacuum and harsh chemical environments.

Although devices have been made by scaling down existing microelectromechanical systems (MEMS), the workhorse methods and materials of MEMS technology are not universally well suited to the nanoscale. Ultra-small silicon-based systems fail to achieve desired high-Q mechanical resonances owing to dominant surface effects and thermoelastic damping, and limitations in strength and flexibility compromise silicon-based high-performance actuators^{5,6}. On the other hand, the unusual mechanical and electronic properties of carbon⁷ and boron-nitride⁸ nanotubes (including favourable elastic modulus and tensile strength, high thermal and electrical conductivity, and low inter-shell friction of the atomically smooth surfaces^{9,10}) suggest that nanotubes may serve as important NEMS-enabling materials.

Figure 1a shows the conceptual design of the electromechanical rotational actuator. The rotational element (R), a solid rectangular metal plate serving as a rotor plate, is attached transversely to a suspended support shaft. The support shaft ends are embedded in electrically conducting anchors (A1, A2) that rest on the oxidized surface of a silicon chip. The rotor plate assembly is surrounded by three fixed stator electrodes: two 'in-plane' stators (S1, S2) are horizontally opposed and rest on the silicon oxide surface, and the third 'gate' stator (S3) is buried beneath the surface. Four independent (d.c. and/or appropriately phased a.c.) voltage signals, one to the rotor plate and three to the stators, can be applied to

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control the position, speed and direction of rotation of the rotor plate. The key component in the assembly is a single multi-walled carbon nanotube (MWNT), which serves simultaneously as the rotor plate support shaft and the electrical feedthrough to the rotor plate; most importantly it is also the source of rotational freedom.

Our NEMS actuator was constructed using lithographic methods. MWNTs, synthesized by the standard arc technique¹¹, were suspended in 1,2-dichlorobenzene and deposited on degenerately doped silicon substrates covered with 1 μm of SiO_2 . The nanotubes were located with respect to pre-patterned alignment marks on the SiO_2 surface using an atomic force microscope or a LEO 1550 scanning electron microscope (SEM). The remaining actuator components (in-plane rotor plate, in-plane stators, anchors, and electrical leads) were then patterned using electron beam lithography. A single layer of electron beam resist (polymethyl methacrylate, 950,000 relative molecular mass, 5.5% in chlorobenzene) was spun on the samples at 4,000 r.p.m. for 45 seconds, and subsequently baked in air at 150 $^\circ\text{C}$ for 2 hours. The resist was then patterned using NPGS software on a JEOL 6400 SEM, and developed in methyl isobutyl ketone:isopropyl alcohol 1:3 for one minute. Gold (~ 90 nm) with a chromium adhesion layer (~ 10 nm) was then thermally evaporated and lifted off in acetone. The Cr/Au was subsequently annealed at 400 $^\circ\text{C}$ to ensure better electrical and mechanical contact between the Cr and the MWNT. An HF etch was used to remove roughly 500 nm of the SiO_2 surface to provide clearance for the rotor plate once it was rotated by 90 $^\circ$. The conducting Si substrate (typically used as the gate electrode in three-terminal nanotube field-effect devices^{12,13}) here serves as the

gate stator. Figure 1b shows an actuator device prior to etching. Typical rotor plate dimensions were 250–500 nm on a side.

Initial actuator characterization was carried out *in situ* inside the SEM. We found that applying voltages up to 50 V d.c. between the (slightly asymmetric) rotor plate and the gate stator generated a net torque sufficient to visibly rotate the rotor plate (up to 20 $^\circ$ deflection). When the applied voltage was removed, the rotor plate would rapidly return to its original horizontal position. Using a finite analysis program (FEMLAB, a commercially available plug-in for MATLAB) and our actuator geometry together with the measured deflection and applied voltages, we determine for our devices typical 'as produced' effective torsional spring constants of 10^{-15} to 10^{-12} N m. Evaluation of the MWNT shear modulus (assuming a continuum mechanics model¹⁴) necessitates knowledge of the outer radii of the nanotubes. We were able to determine the outer diameter of the MWNTs in our devices to within 20%, and found that they ranged from 10 to 40 nm, which was consistent with high-resolution transmission electron microscopy (TEM) measurements of MWNTs from the same preparation batch. TEM imaging also showed the MWNTs to be of high structural quality, composed of concentrically nested cylindrical tubules with no obvious defects. For a 10-nm-diameter MWNT with an effective length of 2 μm , we estimate a shear modulus of 100 to 300 GPa. These ranges for torsional spring constant and shear modulus overlap those of more direct measurements employing a suspended MWNT subjected to torsional deflection via an atomic force microscope tip^{15,16}.

Although the actuator devices just described have a number of extremely useful characteristics (including predicted torsional oscillator mechanical resonance frequencies of the order of tens to hundreds of megahertz), the strong torsional spring constant effectively prevents large low-frequency angular displacements. For large-displacement operation, we modified the MWNT support shaft in an attempt to exploit the intrinsic low-friction bearing behaviour afforded by the perfectly nested shells of MWNTs^{9,10,17,18}. The modification consists of removing or compromising one or more outer MWNT shells in the region between the rotor plate and the anchors. Several *in situ* methods were used to achieve the modification in the SEM, including reactive-ion etching, application of current through the nanotube to 'blow out' outer nanotube shells^{19,20}, and selective nanotube bond-damage

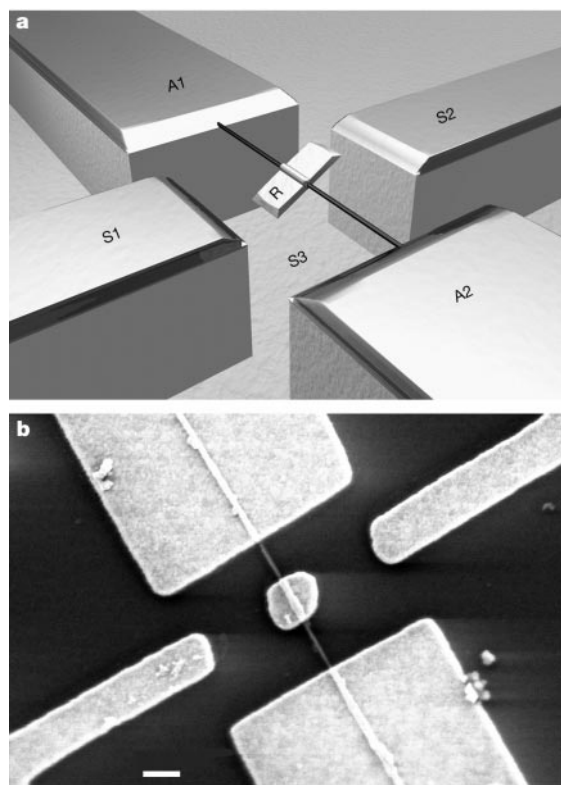


Figure 1 Integrated synthetic NEMS actuator. **a**, Conceptual drawing of nanoactuator. A metal plate rotor (R) is attached to a multi-walled carbon nanotube (MWNT) which acts as a support shaft and is the source of rotational freedom. Electrical contact to the rotor plate is made via the MWNT and its anchor pads (A1, A2). Three stator electrodes, two on the SiO_2 surface (S1, S2) and one buried beneath the surface (S3), provide additional voltage control elements. The SiO_2 surface has been etched down to provide full rotational freedom for the rotor plate. The entire actuator assembly is integrated on a Si chip. **b**, Scanning electron microscope (SEM) image of nanoactuator just prior to HF etching. The actuator components can be identified by comparing this image to **a**. Scale bar, 300 nm.

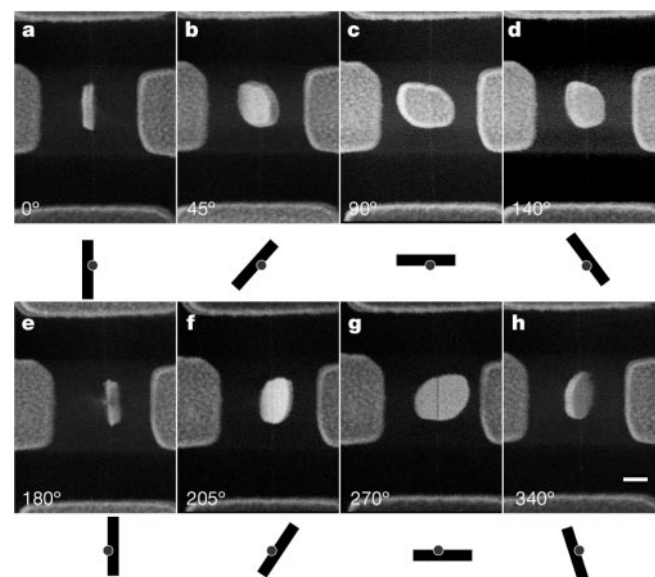


Figure 2 Series of SEM images showing the actuator rotor plate at different angular displacements. The MWNT, barely visible, runs vertically through the middle of each frame. The schematic diagrams located beneath each SEM image illustrate a cross-sectional view of the position of the nanotube/rotor-plate assembly. Scale bar, 300 nm.

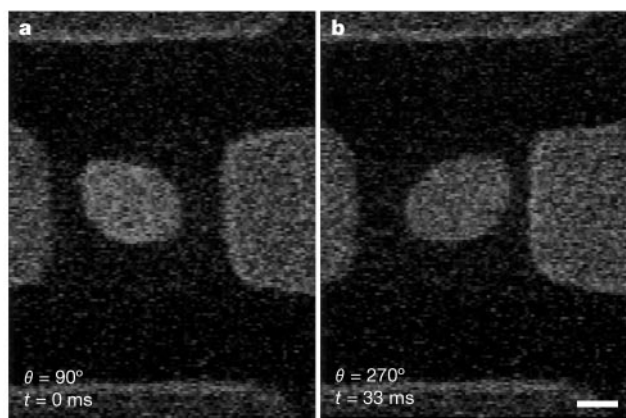


Figure 3 Two SEM images captured from a video recording of an a.c.-voltage-driven actuator ‘flipping’ between the extremal horizontal positions (90° and 270°). The two images are taken one video frame (that is, 33 ms) apart. (See Supplementary Information.) Rapid, large-amplitude rotor plate oscillations could be sustained nearly indefinitely with no noticeable wear or degradation in actuator operation. The MWNT is not visible in these images, but it runs vertically through the middle of each frame. Scale bar, 300 nm.

induced by the SEM electron beam.

A particularly simple yet effective *in situ* MWNT modification method, and the one used on the devices to be described below, was to mechanically fatigue and eventually shear the outer nanotube shells by successive application of very large stator voltages. We found that applied gate stator voltages of order 80 V d.c. would torque the outer nanotube shells past the elastic limit, eventually leading to partial or complete failure of the outer nanotube shells and a resulting dramatic increase in the rotational freedom of the rotor plate. In the ‘free’ state, the rotor plate was still held in position axially by the intact nanotube core shells, but could be azimuthally positioned, using an appropriate combination of stator signals, to any arbitrary angle between 0° and 360°. Once so positioned, the rotor plate nominally remained in place even with all stator voltages reduced to zero, eventually drifting to a vertical (0° or 180°) position only under the charging influence of the SEM imaging electron beam.

Figure 2 shows a series of still SEM images recorded of an actuator device in the free state, being ‘walked’ through one complete rotor plate revolution using quasi-static d.c. stator voltages. The stator voltages, never exceeding 5 V, were adjusted sequentially to attract the rotor plate edge to successive stators. By reversing the stator voltage sequence, the rotor plate rotation could be reproducibly reversed.

Finite frequency operation of the actuator was also performed, using a variety of suitably phased a.c. and d.c. voltage signals to the three stators and rotor plate. In one simple operation mode, we applied out-of-phase common-frequency sinusoidal voltages to stators S1 and S2, a doubled-frequency signal to S3, and a d.c. offset to the rotor plate R; that is, $S1 = V_0 \sin(\omega t)$, $S2 = V_0 \sin(\omega t - \pi)$, $S3 = V_0 \sin(2\omega t + \pi/2)$, $R = -V_0$. Although the resulting spatial and temporal drive forces are actually quite complex, roughly speaking this sequence allowed the rotor plate to be sequentially electrostatically attracted to the next available stator. Using this drive sequence we were reliably able to alternately flip the rotor plate between the extreme horizontal (90° and 270°) positions. Although in principle very high frequency operation should be possible (restricted only by the stripline bandwidth of the leads and, ultimately, inertial effects of the rotor plate), our SEM image capture rate limited direct real-time observations of rotor plate oscillations to frequencies of typically several hertz.

We found that the transitions between the extreme horizontal positions could be made faster than the image video capture rate of 33 ms. Figure 3 shows two images of the actuator, recorded 33 ms apart, showing the rotor plate respectively in the 90° and 270°

positions. We were able to rotationally drive actuators in this fashion for many thousands of cycles, with no apparent wear or degradation in performance. (See Supplementary Information.) In this configuration, the MWNT clearly serves as a reliable, presumably wear-free, NEMS element providing rotational freedom. This characterization was performed in a pressure of 10^{-6} – 10^{-5} torr, although we anticipate reliable operation at higher pressures. We note that our actuator is the first true MWNT-based NEMS device, in that it fully integrates electronic control and mechanical response. This distinguishes it from previous related MWNT-based mechanical devices which require relatively large and complex external control systems (such as piezo-driven manipulators) to achieve operation^{15–18,21}.

Our MWNT-based actuators have obvious MEMS/NEMS applications potential. The rotational metal plate could serve as a mirror, with obvious relevance to ultra-high-density optical sweeping and switching devices (our total actuator size is just at the limit of visible light focusing). The rotating plate could also serve as a paddle for inducing and/or detecting fluid motion in microfluidics systems, as a gated catalyst in wet chemistry reactions, as a bio-mechanical element in biological systems, or as a general (potentially chemically functionalized) sensor element. It is also possible that the charged oscillating metallic plate could be used as a transmitter of electromagnetic radiation. Using methods to align nanotubes, it should be possible to fabricate arrays of orientationally ordered nanotube-based actuators on substrates. □

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Epitaxial self-assembly of block copolymers on lithographically defined nanopatterned substrates

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Parallel processes for patterning densely packed nanometre-scale structures are critical for many diverse areas of nanotechnology. Thin films of diblock copolymers^{1–11} can self-assemble into ordered periodic structures at the molecular scale (~5 to 50 nm), and have been used as templates to fabricate quantum dots^{1,2}, nanowires^{3–5}, magnetic storage media⁶, nanopores⁷ and silicon capacitors⁸. Unfortunately, perfect periodic domain ordering can only be achieved over micrometre-scale areas at best^{12,13} and defects exist at the edges of grain boundaries. These limitations preclude the use of block-copolymer lithography for many advanced applications¹⁴. Graphoepitaxy^{12,15}, in-plane electric fields^{3,16}, temperature gradients¹⁷, and directional solidification^{14,18} have also been demonstrated to induce orientation or long-range order with varying degrees of success. Here we demonstrate the integration of thin films of block copolymer with advanced lithographic techniques to induce epitaxial self-assembly of domains. The resulting patterns are defect-free, are oriented and registered with the underlying substrate and can be created over arbitrarily large areas. These structures are determined by the size and quality of the lithographically defined surface pattern rather than by the inherent limitations of the self-assembly process. Our results illustrate how hybrid strategies to nanofabrication allow for molecular level control in existing manufacturing processes.

In epitaxial assembly of block-copolymer films, molecular-level control over the precise size, shape and spacing of the ordered

domains is achieved by specifying the volume fraction of each block and the length of the chains¹⁹. Lithographic patterning of surfaces provides the following desirable attributes of top-down fabrication techniques to our hybrid strategy: the ability to pattern over truly macroscopic areas, scalability and manufacturability, and placement of each domain, including registration and overlay, with nanometre precision. Epitaxial assembly contrasts with previous experimental studies that describe directed or guided assembly of lamellar domains on chemically striped substrates^{20–22}. Even the highest-quality films in previous studies contained a high density of defects of unknown origin, and were quantified at best by a statistical degree of orientation²⁰. Previous molecular simulation and theoretical studies also describe directed or guided orientation and ordering of cylindrical²³ and lamellar^{24,25} domains on chemically nanopatterned substrates, but are limited in their predictive capabilities concerning epitaxial assembly owing to the finite size, periodic boundary conditions or reduced dimensionality of the analysis.

Figure 1 illustrates the two-step process used to prepare well-defined nanopatterned surfaces. Photoresist was patterned with alternating lines and spaces with periods between 45 and 55 nm using extreme ultraviolet interferometric lithography²⁶ (EUV-IL), and the pattern in the photoresist was transferred to an underlying self-assembled monolayer (SAM) by chemical modification of the SAM. The dimensions of the areas patterned with EUV-IL were approximately 50 μm by 400 μm , although the areas over which the patterns in the resist were perfect were smaller. The period of the chemical surface pattern was known from the design of the EUV-IL mask, and was confirmed by measuring the period of the patterned photoresist. Following removal of the remaining photoresist, a 60-nm film of symmetric lamella-forming poly(styrene-block-methyl methacrylate) ((PS-*b*-PMMA), 104 kg mol^{–1}, lamellar period approximately 48 nm) was spin-coated and annealed on the chemically patterned surface. The PMMA block preferentially wet the SAMs that were chemically modified to contain polar groups²⁷, and the other regions exhibited neutral wetting behaviour by the block copolymer. The domain structure of the block-copolymer film after annealing was imaged using scanning electron microscopy (SEM). The two-step process allows for unprecedented characterization of the chemically nanopatterned surfaces. Future applications may benefit from or require the synthesis of sensitive and chemically specific imaging layers that change functionality directly upon lithographic exposure.

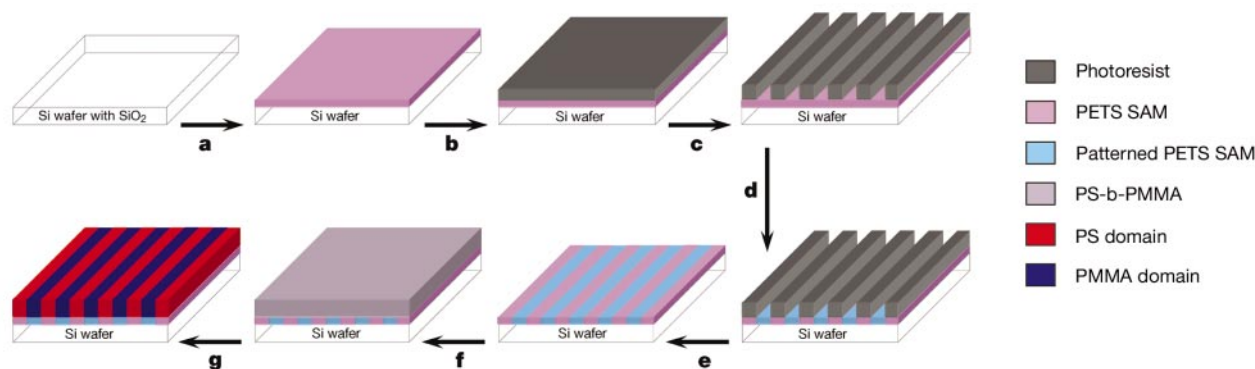


Figure 1 Schematic representation of the strategy used to create chemically nanopatterned surfaces and investigate the epitaxial assembly of block-copolymer domains. **a**, A SAM of PETS was deposited on a silicon wafer. **b**, Photoresist was spin-coated on the SAM-covered substrate, and **c**, patterned by EUV-IL with alternating lines and spaces of period L_s . **d**, The topographic pattern in the photoresist was converted to a chemical pattern on the surface of the SAM by irradiating the sample with soft X-rays in the presence of oxygen. **e**, The photoresist was then removed with repeated solvent

washes. **f**, A symmetric, lamella-forming PS-*b*-PMMA copolymer of period L_0 was spin-coated onto the patterned SAM surface and **g**, annealed, resulting in surface-directed block-copolymer morphologies. Chemically modified regions of the surface presented polar groups containing oxygen and were preferentially wetted by the PMMA block, and unmodified regions exhibited neutral wetting behaviour by the block copolymer.

If the period of the chemically patterned SAM, L_s , was equal to the period of the lamellar spacing of the block copolymer, L_o , ($L_s = L_o = 48$ nm), then the lamellar domains in the block-copolymer films were oriented perpendicularly to the substrate and were perfectly registered with the chemical pattern of the SAM. The largest continuous area over which the chemical surface patterns were defect-free was larger than $8\text{ }\mu\text{m}$ by $5\text{ }\mu\text{m}$. Figure 2a shows a representative top-down image of the registered and perfect long-range order of the overlying block-copolymer film over a $5\text{ }\mu\text{m}$ by $5\text{ }\mu\text{m}$ area. The longest linear distances over which the ordering of the block-copolymer film was perfect along a single lamellar domain (parallel to the pattern) and across the lamellar structure (perpendicular to the pattern) were approximately 400 and $50\text{ }\mu\text{m}$, respectively, and corresponded to the dimensions of the patterned area.

We could correlate the defects in the ordering of the block-copolymer film, when they are present, directly to defects in the patterning of the photoresist and therefore to defects in the chemical pattern of the SAM. In our EUV-IL system, for example, reproducible fringes in the patterned photoresist appeared near the edges of the patterned area owing to diffraction effects. The defects consisted of stripes of slightly over- and underexposed material that diminished in intensity with distance from the edge of the patterned region (Fig. 2b). Defects in the domain structure of the block-copolymer films (Fig. 2c) occurred at the same locations as defects in the patterns of photoresist. In overexposed areas (see area (2) in Fig. 2b), the photoresist structures were discontinuous and lacked integrity. The structure of the block-copolymer film over these predominantly chemically modified and therefore polar regions (see area (2) in Fig. 2c) consisted of parallel lamellae with the PMMA block wetting the surface. In underexposed areas (see area (3) in Fig. 2b) the photoresist was not cleared completely between the patterned line structures and pattern transfer to the SAM did not occur. The structure of the block-copolymer film on these areas (see

area (3) in Fig. 2c) consisted of unregistered perpendicular lamellae with no long-range order. This structure was consistent with that observed on unpatterned regions (see areas labelled (1) in Fig. 2b and c).

The natural grain size or length scale of ordering of lamellae, as observed on homogeneous neutral areas, was about $2L_o$. This length scale is significantly less than that in analogous sphere-forming and cylinder-forming block-copolymer films because of the reduced number of degrees of freedom available for the lamella-forming system to organize. Our approach, without optimization of interfacial interactions or processing conditions, resulted in registration and long-range ordering at length scales two to three orders of magnitude larger than the natural grain size. On the basis of these results, we believe that there is no inherent limitation on the size of the registered and ordered array of domains that can be created by epitaxial assembly on lithographically defined chemically nanopatterned substrates.

Distinctly different types of registration and ordering defects of lamellar domains were observed if L_s was slightly smaller or larger than L_o . Top-down images of the in-plane domain structure of PS-*b*-PMMA ($L_o = 48$ nm) and corresponding Fourier transform analysis of the images are shown in Fig. 3 for $L_s = 45, 47.5, 50, 52.5$ and 55 nm. The film thickness was 60 nm, or approximately $1.2L_o$, for all samples, and the images correspond to the best $3\text{ }\mu\text{m}$ by $3\text{ }\mu\text{m}$ area of the sample over which the chemical surface pattern was defect-free. For $L_s = 45$ nm, the lamellae were compressed but were for the most part registered and epitaxial to the surface pattern. The sharp peaks in the Fourier transform analysis correspond to a period of less than L_o . Occasionally the mismatch between $L_s < L_o$ led to the formation of pairs of defects.

For $L_s = 47.5$, $L_s \cong L_o$ and the registration and ordering of the block-copolymer domains was perfect over the entire imaged area. The Fourier transform analysis reveals only two very sharp peaks at

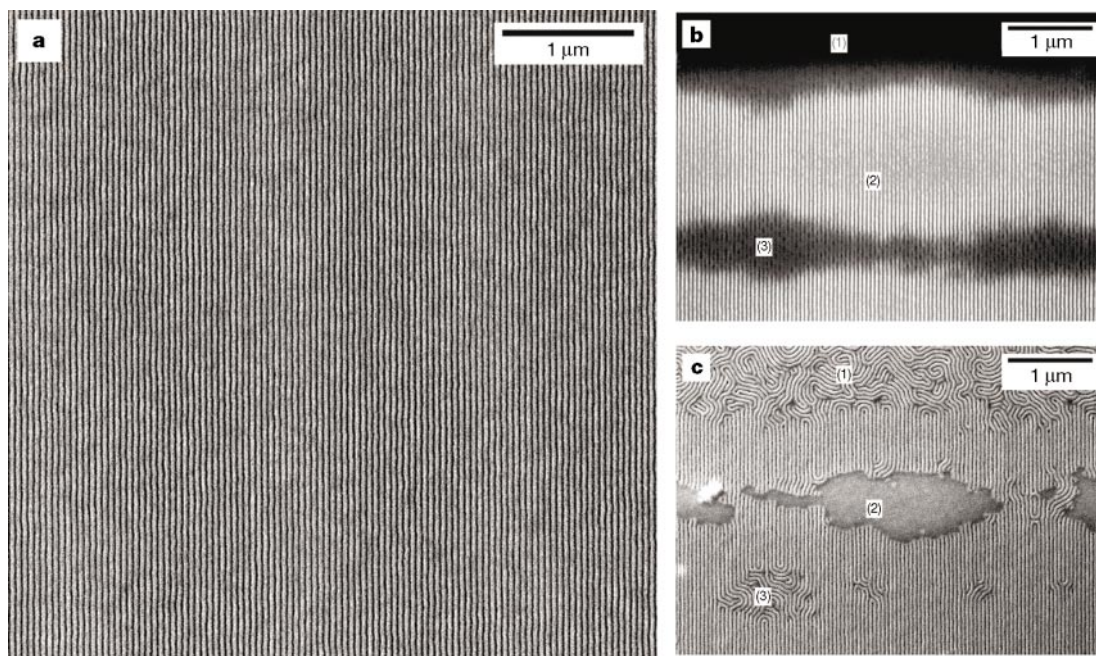


Figure 2 Top-down SEM images of photoresist and PS-*b*-PMMA copolymer ($L_o = 48$ nm, film thickness of 60 nm) patterns. In the SEM images of the PS-*b*-PMMA copolymer the light and dark regions were PS and PMMA domains, respectively. **a**, The perfect epitaxial ordering of a block-copolymer pattern extended over a $5\text{ }\mu\text{m}$ by $5\text{ }\mu\text{m}$ area. In this case, the SAM surface was chemically patterned with a period of $L_s = 47.5$ nm that matched L_o . **b**, Top-down SEM image of the photoresist pattern ($L_s = 47.5$ nm) at the edge of the region exposed using EUV-IL, and **c**, top-down SEM image of the morphology of a block-copolymer film overlying a chemically nanopatterned surface that was

prepared (as in Fig. 1) using a photoresist pattern similar to that shown in **b**. To allow for comparison between samples, the exposure doses during EUV-IL and chemical modification steps were controlled to within 2%. Regions (1), (2) and (3) in **b** delineate unpatterned, overexposed, and underexposed areas of the photoresist, respectively. Regions (1), (2) and (3) in **c** show the block-copolymer morphologies observed over corresponding areas. Lamellae were oriented perpendicularly without long-range order on the unpatterned (1) and underexposed (3) regions. In the overexposed (2) regions the lamellae adopted a morphology that was parallel to the surface.

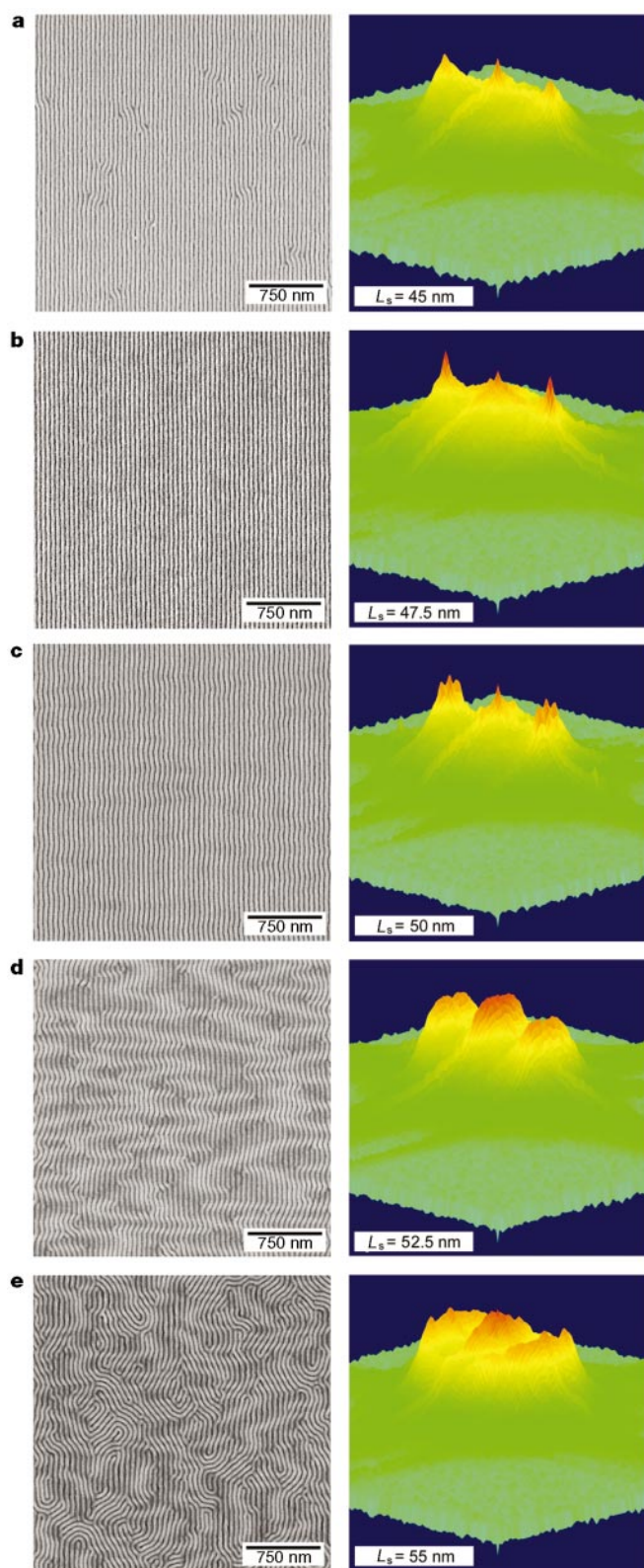


Figure 3 Top-down SEM images and corresponding Fourier transform analysis of PS-b-PMMA copolymer films ($L_0 = 48$ nm, film thickness of 60 nm) on chemically nanopatterned surfaces. The surfaces had essentially perfect chemical patterns with periods, L_s , of: **a**, 45; **b**, 47.5; **c**, 50; **d**, 52.5; and **e**, 55 nm. The area of each SEM image corresponds to a $3\ \mu\text{m}$ by $3\ \mu\text{m}$ region of block-copolymer domains.

a characteristic period of 47.5 nm. For $L_s > L_0$, the top-down images revealed two distinct mechanisms of defect formation in the ordering of lamellar domains: (1) the formation of herringbone patterns and (2) unregistered lamellae with a period of L_0 that were aligned to varying degrees with the axis of the surface pattern. As the difference between L_s and L_0 increased, the angle between the herringbone pattern and the axis of the surface pattern, θ_{hb} , increased, and the fraction of lamellae registered and the degree of alignment of the unregistered lamellae with the surface pattern decreased. The herringbone morphology is particularly evident in the Fourier transform analysis of the sample with $L_s = 50$ nm. Six peaks appeared at periods in the neighborhood of L_0 (Fig. 3c). The peaks associated with structures perpendicular to the axis of the surface pattern had a period slightly greater than L_0 . The off-axis peaks corresponded to structures with a period of L_0 , and the location of the peaks revealed a single value of $\theta_{\text{hb}} \approx 8^\circ$. For $L_s = 52.5$ and 55 nm, the herringbone morphology was also observed but a much greater fraction of the lamellae fell out of registry with the surface pattern (Fig. 3d, e). In addition, multiple values of θ_{hb} were derived from the coordinates of local peaks in the Fourier transform analysis. For $L_s = 55$ nm, for example, the most intense peaks yielded values of θ_{hb} of ~ 10 and 25° .

Cross-sectional SEM images in Fig. 4 show that out-of-plane defects in ordering and orientation also occur for $L_s > L_0$. Lamellae are oriented perpendicular to the substrate for block-copolymer films on neutral homogenous (Fig. 4a) and for epitaxially ordered films if $L_s = L_0$ (Fig. 4b). The lamellae were tilted with respect to the surface normal, however, for films where $L_s > L_0$ (Fig. 4c). Tilt also introduces grain-boundary defects. Adoption of the herringbone and tilted morphologies are mechanisms by which the interfacial area between the PS and PMMA blocks and the polar stripes of the surface pattern are minimized and maximized, respectively. Consistent with the limitations of previously published models discussed earlier, tilted lamellae have been predicted by molecular

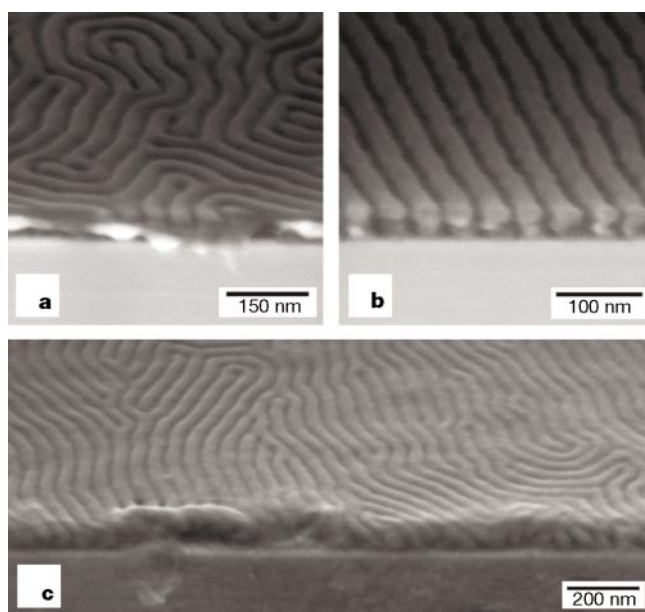


Figure 4 Cross-sectional SEM images of PS-b-PMMA films ($L_0 = 48$ nm, thickness 60 nm) on unpatterned and chemically nanopatterned surfaces. The samples were cleaved under cryogenic conditions and imaging was performed on a stage tilted at 60° . **a**, Lamellae were oriented perpendicularly with no long-range order on unpatterned regions of the surface. **b**, Lamellae were oriented perpendicularly with epitaxial ordering on surfaces for which $L_s = L_0$. **c**, Lamellae exhibited both herringbone and tilt defects when the surface pattern ($L_s = 55$ nm) was greater than L_0 .

simulation²⁴ and theory²⁵ but the herringbone morphologies have not.

These results clarify distinctions between epitaxial and surface-directed ordering of block-copolymer films. Orientation, registration and defect-free order required commensurability between the period of the surface pattern and the block copolymer within just a few per cent. If the periods of the surface and block copolymer agreed within approximately 10% ($L_s = 45, 50$ and 52.5 nm), the morphology of the block-copolymer film was surface-directed but not perfect. This constraint is a function of many parameters, including the polymer chemistry, film thickness and the magnitude of the surface and interfacial interactions.

There are essential aspects of this integrated fabrication strategy combining advanced lithography and self-assembly that enhance its potential technological impact. It is not limited to the use of PS-*b*-PMMA, but is applicable to other systems provided sufficient contrast can be achieved in the interfacial interactions between the patterned substrates and the blocks of the copolymer. Lithographic patterning of the substrate is simplified because the two-dimensional projections of desired domain structures in ordered block-copolymer films correspond to diffraction patterns that can be produced with multiple beam interferometry. Epitaxial assembly can easily be extended to cylinder-forming block copolymers that present immediate opportunities for the fabrication of addressable arrays for applications such as magnetic storage media. □

Methods

A SAM was deposited on a silicon wafer by immersing the sample in a 0.1 vol.% solution of phenylethyltrichlorosilane (PETS) in toluene for 1 h. A ~ 55 -nm film of photoresist (PMMA, 960 kg mol⁻¹) was then spin-coated on the SAM-covered substrate and baked at 160 °C for 1 min to remove residual solvent. The PMMA was patterned using EUV-IL ($\lambda = 13.4$ nm, Center for NanoTechnology (CNTech), University of Wisconsin) in which achromatic radiation passed through a transmission membrane mask that consisted of a rectangular ($\sim 50 \mu\text{m}$ by $400 \mu\text{m}$) opaque region, with grating structures of period p on opposing sides of the opaque region. The mask was fabricated by patterning chrome on a SiN membrane using electron beam lithography and reactive ion etching techniques. The first-order diffracted beams from the gratings interfere with one another in the shadow of the opaque region of the mask to generate an interference pattern with a period of $p/2$. We targeted 45 to 55 nm period lines and spaces, so the periods of the grating structures written on the mask were 90 to 110 nm. The gap between the mask and the sample was typically 0.35 mm, and the exposure tool offers exceptional depth of focus. After exposure, the PMMA was developed using standard lithographic procedures and blown dry in a stream of N₂. The period of the photoresist pattern was determined from SEM images by averaging over ten periods of lines. The topographic pattern in the photoresist was transferred to a chemical pattern on the surface of the SAM by irradiating the sample with soft X-rays ($\lambda = 1.1$ nm, ES-1 beamline at the CNTech) in the presence of oxygen (1 torr of air). The SAM in regions that were not covered by photoresist underwent photoelectron-induced reactions that led to the incorporation of polar, oxygen-containing functional groups on the surface. The remaining PMMA photoresist was stripped from the sample using chlorobenzene. Films of controlled thickness (~ 60 nm) of PS-*b*-PMMA (104 kg mol⁻¹, lamellar period of 48 nm) were deposited on chemically patterned substrates via spin-coating from dilute solutions in toluene. The films were then annealed for over 7 days at 190 °C. After annealing the domain structure of the block-copolymer film was imaged using a LEO 1550 VP field emission SEM.

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Carbon solubility in olivine and the mode of carbon storage in the Earth's mantle

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The total amount of carbon in the atmosphere, oceans and other near-surface reservoirs is thought to be negligible compared to that stored in the Earth's mantle^{1–3}. Although the mode of carbon storage in the mantle is largely unknown, observations of microbubbles on dislocations in minerals from mantle xenoliths has led to the suggestion that carbon may be soluble in silicates at high pressure^{4,5}. Here we report measurements of carbon solubility in olivine, the major constituent of the upper mantle, at pressures up to 3.5 GPa. We have found that, contrary to previous

expectations, carbon solubility in olivine is exceedingly low—of the order of 0.1 to 1 parts per million by weight. Together with similar data for pyroxenes, garnet and spinel, we interpret this to imply that most carbon must be present as a separate phase in the deeper parts of the upper mantle, probably as a carbonate phase^{6,7}. Large-scale volcanic eruptions tapping such a carbonate-bearing mantle reservoir might therefore rapidly transfer large amounts of carbon dioxide into the atmosphere, consistent with models that link global mass extinctions to flood basalt eruptions via a sudden increase in atmospheric carbon dioxide levels^{8–11}.

Carbon solubility in olivine is crucial for understanding the dynamics of the carbon exchange between the mantle and near-surface reservoirs. Even a solubility of the order of a few hundred parts per million (p.p.m.) by weight would be sufficient to incorporate all carbon in the upper mantle in olivine. If carbon were present in a dilute solid solution in olivine, mobilizing large amounts of carbon from the mantle and transferring it within short timescales to the atmosphere would be impossible. Earlier reports¹² of high carbon concentrations dissolved in olivine ($(\text{Mg,Fe})_2\text{SiO}_4$) have not been confirmed by later studies^{13–15}.

Previous attempts to measure carbon solubility in olivine were probably hampered by contamination problems¹⁵ and by slow diffusion coefficients¹⁴, which make it difficult to diffuse carbon into pre-existing olivine crystals. We have overcome these problems by growing carbon-saturated olivine crystals from a carbonatite melt. Small amounts of carbonatite melts are common in the Earth's mantle^{7,16}. Moreover, we used starting materials in our experiments that were isotopically enriched to contain more than 99% in the isotope ^{13}C , which has a natural abundance of only 1%. The unusual isotopic composition of this carbon allows it to be easily distinguished from any contamination with normal isotopic composition.

Starting material in our experiments was a stoichiometric mixture of MgO and SiO_2 together with 20 wt% of $\text{Na}_2^{13}\text{CO}_3$. This mixture was sealed together with about 1 wt% of water into platinum-rhodium capsules. Crystallization experiments were carried out in a piston cylinder apparatus at 1,200 °C and 1 to 3.5 GPa, with run durations of several days. Talc Pyrex cells with a graphite heater were used for the experiments. The pressures reported include a 20% friction correction. The redox conditions during the experiments were not strictly controlled, but the reaction of the graphite heater with the water released from the talc probably yielded an oxygen fugacity close to the Ni–NiO buffer. Run products consisted of clear, inclusion-free euhedral forsterite (Mg_2SiO_4) crystals up to several hundred micrometres in size embedded in a matrix of fine-grained carbonates quenched from the carbonatite melt. Infrared spectra of the carbonates recovered after the experiments demonstrated that the carbon in the sample capsule was ^{13}C with only minor impurities of ^{12}C (Supplementary Fig. 1). All carbonates were removed by treatment with dilute hydrochloric acid. Forsterite crystals were mounted on glass slides with carbon-free ceramic glue. Samples were polished using 3 μm diamond powder and gold-coated. Carbon contents were measured with a

Cameca ims 6f ion probe using a 2 nA, 10 kV Cs^+ primary beam focused on a 5 μm spot. All pumps in the vacuum system were oil-free, and yielded a vacuum in the 10^{-10} torr range. In addition, a liquid-nitrogen cold trap was used to suppress further any carbon background in the vacuum. The surfaces of all samples were cleaned by ion bombardment before commencing data collection.

Despite all of these efforts, the measured carbon was dominated by the ^{12}C isotope. However, in contrast to samples with isotopically normal carbon contents, where a $^{13}\text{C}/^{12}\text{C}$ ratio of $0.994\% \pm 0.002\%$ ($n = 8$) was measured, the synthetic forsterite crystals yielded elevated $^{13}\text{C}/^{12}\text{C}$ ratios up to 2%. These elevated isotopic ratios were found to be stable in several depth profiles. Assuming that any contamination has normal isotopic composition, it was possible to calculate the ^{13}C count rate arising from the ^{13}C content intrinsic to the sample. Synthetic forsterite crystals ion-implanted with a known dose of ^{13}C were used as reference samples. They yielded a relative sensitivity factor for $^{13}\text{C}/^{28}\text{Si}$ of 0.481 ± 0.043 ($n = 7$), which was used to quantify the carbon content of the samples. In addition, three tholeiite glasses doped with variable amounts of ^{13}C and natural spurrite ($\text{Ca}_5\text{CO}_3(\text{SiO}_4)_2$) samples were also measured. Estimates of carbon contents using these standards are all within one order of magnitude of the values obtained from the implant standards, thereby eliminating the possibility of any gross systematic error.

Experimental results are given in Table 1 and Fig. 1, with additional analytical details in Supplementary Table 1. Carbon solubility in olivine is exceedingly low, of the order of 0.1 p.p.m. by weight. Even if the data in Fig. 1 were extrapolated to 10 GPa, corresponding to the base of the upper mantle, solubilities would only reach about 1 p.p.m. Preliminary measurements of carbon solubility in enstatite (MgSiO_3), diopside ($\text{CaMgSi}_2\text{O}_6$), pyrope ($\text{Mg}_3\text{Al}_2(\text{SiO}_4)_3$) and spinel (MgAl_2O_4) synthesized at 900–1,300 °C and 1–6 GPa yielded similarly low carbon solubilities of the order of 0.1 to 1 p.p.m. by weight (Table 2). These measurements were carried out using essentially the same experimental methods as outlined above, with the only significant difference that the ion probe data were calibrated against a glass standard. Since secondary ion yields are matrix dependent and since this standard is not as perfectly matched to the samples as the ion-implanted olivine standard used for the olivine measurements, the data in Table 2 should only be regarded as preliminary and systematic errors by a factor of two are possible. The order of magnitude of carbon solubility, however, is certainly correct.

Considering that a plausible mechanism for the incorporation of carbon in silicates is the direct substitution of C^{4+} for Si^{4+} on tetrahedral sites, it is not surprising that the carbon solubilities in various silicates are similar, since the geometry of the Si^{4+} site hardly varies among common silicate structures¹⁷. This mechanism would also imply that carbon solubility in equilibrium with a carbonatite

Table 1 Experimental results on carbon solubility in forsterite

Run no.	T (°C)	P (GPa)	Duration (h)	Carbon (wt p.p.m.)
CO3	1,200	1.5	68	0.25 ± 0.02
				0.15 ± 0.02
				0.15 ± 0.02
				0.14 ± 0.02
CO5	1,200	3.5	71	0.54 ± 0.06
				0.39 ± 0.04
				0.29 ± 0.04
CO6	1,200	2.0	71	0.34 ± 0.04
CO7	1,200	1.0	34	Not detected

Each carbon concentration refers to a single analysis on an individual crystal. Errors are one standard deviation. Concentrations in wt p.p.m. are recalculated for carbon of normal isotopic composition. For additional analytical details, see Supplementary Table 1.

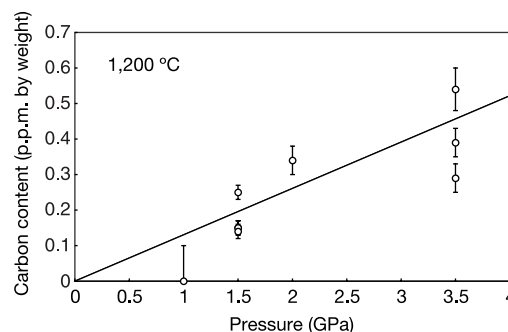


Figure 1 Carbon solubility in forsterite as a function of pressure at 1,200 °C. Data points represent analyses of individual crystals. Errors correspond to one standard deviation. The straight line is only a guide for the eye.

Table 2 Preliminary data on carbon solubility in mantle minerals

Run no.	T (°C)	P (GPa)	Duration (h)	Carbon (wt p.p.m.)
E9 (enstatite)	900	1.5	96	4.7 3.0 Not detected
Di9 (diopside)	900	1.5	168	0.5 0.4
Py6/5 (pyrope)	1,300	6	10	2.1 1.9
Sp24 (spinel)	1,100	1.5	168	Not detected

Each carbon concentration refers to a single analysis on an individual crystal. Concentrations in wt p.p.m. are recalculated for carbon of normal isotopic composition. For errors see text. Sample E9 appears to be inhomogeneous and may contain submicroscopic inclusions so that the data should be considered as an upper limit of carbon solubility only. Experiment Py6/5 was carried out in a multi-anvil apparatus, all other experiments were conducted in a piston cylinder press.

melt is independent of oxygen fugacity, since the oxidation state of carbon in both coexisting phases is the same.

Bulk mantle carbon abundances have been estimated to be of the order of several hundred to more than 1,000 p.p.m. by weight². For the depleted MORB source, some very low values of 72–270 p.p.m. by weight of CO₂ have recently been derived from the analyses of undegassed melt inclusions^{18–19}. Abundances in undepleted mantle sources should be much higher. By comparing any of these numbers with the measured carbon solubilities it is evident that this carbon cannot be present in olivine or any other major mineral of the upper mantle. Instead, it must reside in a separate carbon-rich phase. Extensive experimental research in the 1970s has demonstrated that only CO₂-rich fluids and carbonates are plausible phases for storing carbon in the mantle^{6,7}, if it is not dissolved in silicate minerals. CO₂ reacts with olivine at pressures above 2–3 GPa to form carbonates according to reactions of the type $\text{MgCO}_3 + \text{MgSiO}_3 = \text{Mg}_2\text{SiO}_4 + \text{CO}_2$. Therefore, carbonates appear to host more than 90% of the carbon present in the deeper part of the upper mantle. This situation is very different from that observed for hydrogen, another volatile element, where it has been demonstrated that the entire mantle reservoir can be incorporated in nominally anhydrous minerals such as olivine^{20,21}.

Our results have profound implications for the dynamics of the global carbon cycle. If traces of carbon were stored as a solid solution in olivine, releasing large quantities of carbon to the atmosphere would be virtually impossible on short timescales because of the slow diffusion coefficients of carbon in olivine, and because during normal melting events, only a little olivine is dissolved in the magma. For the same reasons, the exchange of hydrogen between the Earth's mantle and the hydrosphere is slow and the volume of the oceans has changed little over the last three billion years. However, carbonates are easily mobilized during even low degrees of partial melting, and upon decompression below 2.5 to 3.5 GPa they decompose and release CO₂ (refs 6, 7). Moreover, geochemical evidence suggests a highly inhomogeneous distribution of carbon throughout the mantle^{2,16,18}. It is therefore conceivable that a large melting event in a carbonate-rich part of the deeper mantle could rapidly transfer large amounts of carbon dioxide into the atmosphere. This is consistent with models that link extinction events, such as the event at the Triassic–Jurassic boundary, to flood basalt eruptions via a sudden increase of global CO₂ levels^{8–10}. □

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Reflection signature of seismic and aseismic slip on the northern Cascadia subduction interface

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At the northern Cascadia margin, the Juan de Fuca plate is underthrusting North America at about 45 mm yr⁻¹ (ref. 1), resulting in the potential for destructive great earthquakes^{2,3}. The downdip extent of coupling between the two plates is difficult to determine because the most recent such earthquake (thought to have been in 1700)⁴ occurred before instrumental recording. Thermal and deformation studies⁵ indicate that, off southern Vancouver Island, the interplate interface is presently fully locked for a distance of ~60 km downdip from the deformation front. Great thrust earthquakes on this section of the interface (with magnitudes of up to 9)^{4,5} have been estimated to occur at an average interval of about 590 yr (ref. 3). Further downdip there is a transition from fully locked behaviour to aseismic sliding (where high temperatures allow ductile

deformation), with the deep aseismic zone exhibiting slow-slip thrust events⁶. Here we show that there is a change in the reflection character on seismic images from a thin sharp reflection where the subduction thrust is inferred to be locked, to a broad reflection band at greater depth where aseismic slip is thought to be occurring. This change in reflection character may provide a new technique to map the landward extent of rupture in great earthquakes and improve the characterization of seismic hazards in subduction zones.

The surface coverage of regional reflection profiles probing the deep structure of the Cascadia subduction zone north of the Olympic peninsula is shown in Fig. 1. New images formed by processing the 1998 SHIPS⁷ data significantly extend the study area and permit mapping variations in the reflection nature of the interplate interface, from the deep-sea deformation front to where the megathrust interface reaches the forearc mantle corner. Despite considerable variation in the reflection character of the subduction thrust on the seismic lines, there is a consistent downdip change from a thin (<2 km) reflection zone offshore to a broad reflection band (E-layer) some 5 to 7 km thick, inland of the west coast of Vancouver Island (Fig. 2).

In Fig. 3, we show two reflection transects across the northern Cascadia subduction zone formed by combining images from four reflection campaigns. The NE-trending transect (Fig. 3a; red lines in Fig. 1) extends from the deformation front to the east coast of Vancouver Island. The SE-trending transect (Fig. 3b; green lines in Fig. 1) extends from just offshore of Vancouver Island, where it crosses the SW-NE transect, to the eastern end of the Juan de Fuca Strait. Most of the reflection images in Fig. 3 are shown superimposed on a colour background representing tomographic P-wave velocities. The partially imaged interplate interface at the western half of the NE-trending transect (line 85-01 in Fig. 3a) is mostly characterized by a single reflection (as in Fig. 2a). The interplate interface appears to shallow landward of 40 km on the line 85-01 time section (Fig. 3a). This is a pull-up effect caused by a large lateral

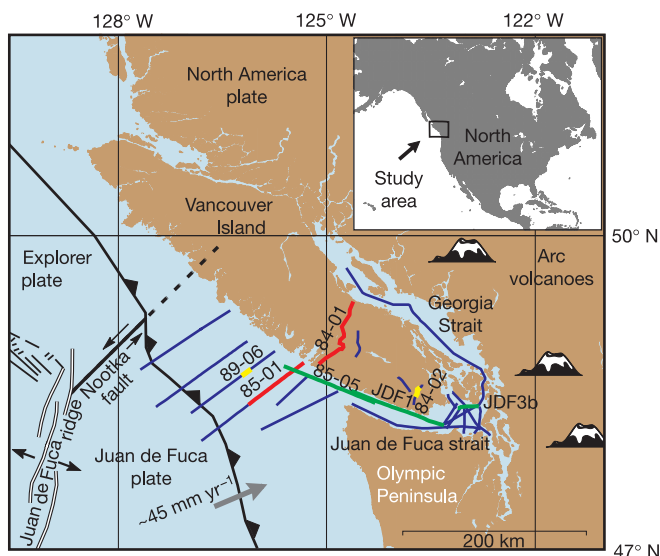


Figure 1 Simplified tectonic map of the northern Cascadia subduction zone. Purple, green and red lines are regional seismic reflection profiles. Four lines located on Vancouver Island were collected in 1984 as part of the Lithoprobe project. Nine lines found offshore of Vancouver Island were shot in 1985 for the Frontier Geoscience project of the Geological Survey of Canada, and in 1989 for the Geological Survey of Canada as part of the Ocean Drilling Program site survey. Profiles in the Strait of Georgia and Strait of Juan de Fuca were collected in 1998 during the SHIPS (Seismic Hazards Investigations in Puget Sound) experiment⁷. Short yellow lines show the surface location of the two details in Fig. 2. Red and green lines indicate the surface trace of the two reflection transects shown in Fig. 3. The inset shows the location of the tectonic map with respect to North America.

velocity change in the top 3–4 km of the crust. The subducting slab in this area is likely to have a smoother and more uniformly landward dipping geometry, similar to the one observed in Fig. 3b.

At the eastern end of line 85-01, the thrust reflection broadens into the 1.5 to 2 s (~5–7 km) wide E-reflection zone that extends through much of land line 84-01 and then abruptly ends. Reflections from the oceanic Moho are visible intermittently along line 85-01 and at the very western end of line 84-01. The Moho reflection is weak and has a similar shape to that of the megathrust event but is found at a recording time about 1.5 to 2 s later (5–7 km deeper). The E-reflection zone is imaged over the full length of the NW-SE transect (Fig. 3b). At the western end of line 85-05 the E-reflection zone is relatively thin (2–3 km). It gradually thickens landward reaching up to 8 km near the end of line 85-05, and is clearly visible

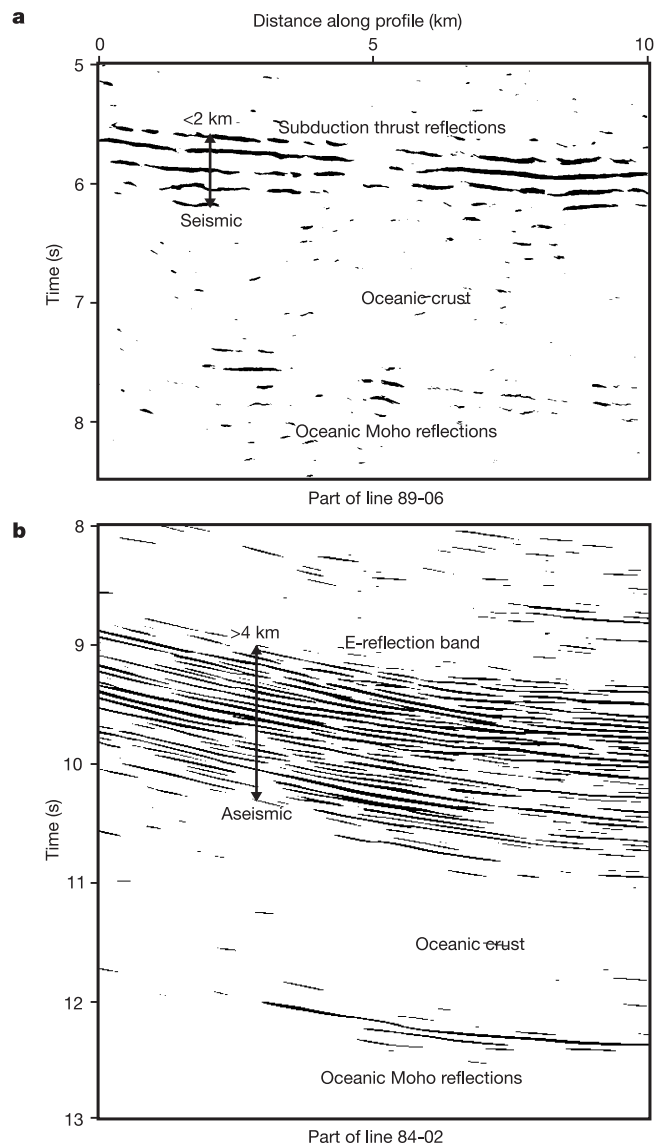


Figure 2 Two details showing the variation in the reflection character at the subduction thrust. **a**, Thin (<2 km) reflection response from the subduction thrust below line 89-06. The thrust is probably represented by a single reflection or a thinner reflection zone (<0.5 km) but the deconvolution was not able to remove the ringing and the stacking was not optimal, passing only the very low part of the signal spectrum. **b**, Thick (>4 km) band of reflections imaged on the most southern Vancouver Island line 84-02 overlies the subducted oceanic crust. In the initial Lithoprobe sections this thick, strong and landward dipping reflection band was named the 'E-layer' because it lies below a series of shallower reflections (A–D)¹⁰. To be compatible with the early interpretations we keep the name but in this work 'E' stands for 'electrically highly conductive'.

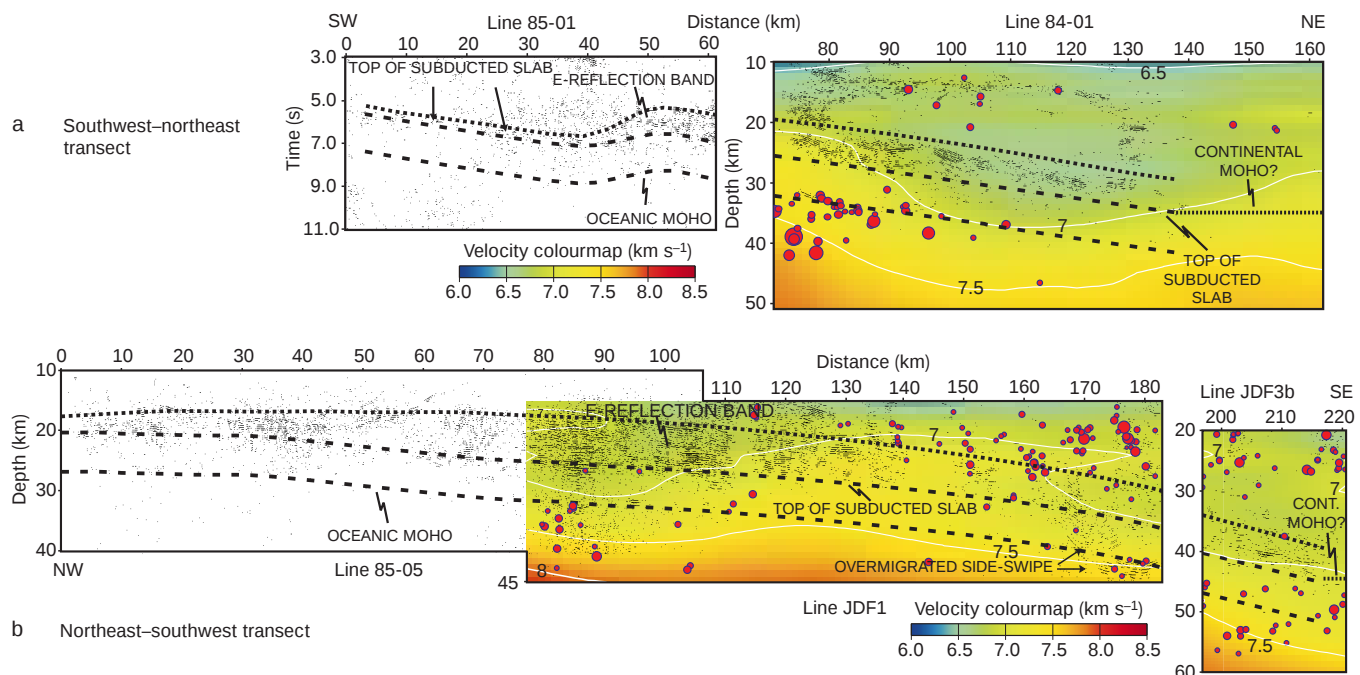


Figure 3 Two reflection transects across the northern Cascadia subduction zone. **a**, The 160-km-long NE-trending transect comprises profiles 85-01 and 84-01. **b**, The 220-km-long SE-trending transect comprises profiles 85-05, JDF1 and JDF3b. The presented images are time-migrated stacks. All except the 1985-01 image were converted to depth along vertical rays. Data from various campaigns were acquired using different equipment and were differently processed, resulting in non-uniform images. Only the depth or time range of interest is shown. P-wave tomographic velocities²⁴, extracted from a 3D velocity volume covering much of the study area, are shown as colour background for profiles 84-01, JDF1 and JDF3b. The tomographic velocities were computed by simultaneously

inverting first P-wave arrivals from both the SHIPS 1998 wide-angle data set⁷ and a selected earthquake data set²⁴. Relocated seismicity from 50-km-wide swaths centred on the profiles is projected on the reflection images and is shown as circles whose size corresponds to earthquake magnitude (1.0–4.6). The ~1-s pull-up structure at about 40–50 km on line 85-01 is caused by a large lateral velocity change at the boundary between the Eocene fossil trench and the Pacific Rim terrain, located further landward. Sediments in the fossil trench are more than 3 km (~2.3 s) thicker than over the neighbouring Pacific Rim terrain, and show significantly lower average velocities (~3 km s⁻¹)²⁸ than the rocks of the Pacific Rim terrain (5.25 km s⁻¹)²⁹.

along lines JDF1 and JDF3b. The oceanic Moho is partially imaged along line 85-05 and at the western end of line JDF1.

The E-reflection band is regionally extensive (Fig. 4a) and where fully developed, it is 5 to 8 km thick. This band is present on all 1998 Juan de Fuca lines except for the easternmost S-trending line, on all 1984 Vancouver Island lines, on the 85-05 offshore line, and at the landward end of a number of other 1985 and 1989 offshore lines. The interpretations proposed for the nature of the E-reflection band can be divided into two classes, structural and nonstructural⁸. In the structural interpretations, the E-reflection zone is represented by interlayered mafic and/or sedimentary rocks^{9,10}, or intensely sheared sediments that trap fluids released from the subducting plate¹¹. In the nonstructural interpretation, the E-reflection zone is caused by thin dipping lenses of high porosity, where the fluid is supplied by dehydration reactions within the subducted oceanic slab¹².

We believe that the E-reflections are related to both shearing and fluids, with the bulk of reflections caused by shearing. Temperature estimates on the megathrust⁵ and fluid-filled porosity estimates within the E-reflection zone^{12,13} lead us further to propose that ductile banding is the prevailing type of deformation in the E-layer shear zone. Laboratory and field studies of rocks from now-exposed deep faults and shear zones show that at depths of 10 to 15 km or more and temperatures above 250 to 350 °C, ductile processes begin to dominate and mylonites are formed¹⁴. The depth at which the E-reflection band is observed is everywhere greater than about 15 km (Fig. 3). The temperature at the base of the E-reflection band at its seaward limit is about 400 °C, increasing to about 500 °C at its landward limit.

Mylonite zones from exhumed ductile shear zones are often as wide as the E-reflection band¹⁵ and are in most cases inferred to

be very reflective even in the absence of water¹⁴. However, the E-reflection band must contain significant fluid-filled porosity to explain its high electrical conductivity^{12,13}, and inferred high Poisson's ratio¹⁶. The fluid paths are probably well interconnected because high tomographic P-wave velocities restrict this porosity to only a few per cent¹². The E-reflection band may represent subducted and metamorphosed sediments. Alternatively, because the obtained tomographic velocities appear to be high for fluid-filled and sheared metasediments, perhaps the shearing cuts up into the overlying ductile forearc crust. The latter process should result in crustal material being transported to greater depths.

The E-reflections appear to end abruptly approximately where the subduction thrust encounters the forearc mantle wedge. For the SW–NE transect the E-reflections end at a depth of about 33 ± 3 km. Early two-dimensional (2D) seismic refraction data provide a depth of 37 ± 1 or 2 km for the continental Moho forming the lid of the forearc mantle wedge¹⁷. The original error estimate on the continental Moho depth should be increased to ± 3 or 4 km, since the new tomography volume shows that the velocity structure is complex and highly three-dimensional. The continental Moho in the area could also be ~6 km shallower than previously interpreted, and therefore in better agreement with our results (Fig. 3a), because the strong wide-angle reflections in the early 2D refraction data recorded on Vancouver Island may well be from the subducting Moho and not from the continental Moho. Recent interpretations^{18,19} show that wide-angle reflections from the continental Moho are very weak in the region of the forearc mantle wedge because mantle here is probably serpentinized and the velocity contrast at the continental Moho is very small. Free slip between the oceanic plate and the stable-sliding serpentinized rocks

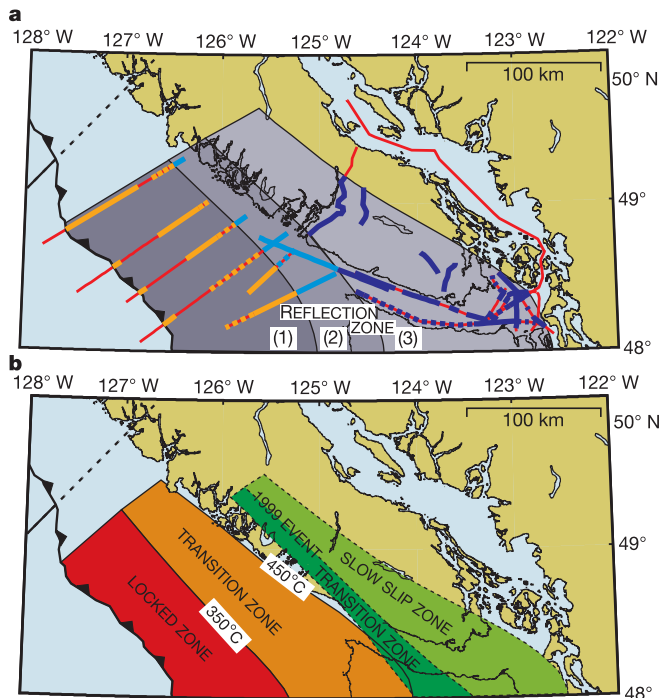


Figure 4 Locked seismogenic, transition, and stable slipping sections of the northern Cascadia subduction interface. Sections inferred from **a**, the extent and character of the subduction thrust reflections and **b**, thermal and dislocation studies⁵, and dislocation modelling of the 1999 slow slip event⁶. **a**, Reflection zone 1 is characterized by thrust reflections less than 2 km thick. Zone 3 has a reflection band immediately above the oceanic plate that is more than 4 km thick. Within zone 2, this reflection band thickens from <2 to >4 km. Three-dimensional geometry of the subducted slab was taken into account when plotting the extent of the reflection zones. Thin red lines are reflection profiles. Overlying thick orange, blue, and purple lines indicate respectively where thin thrust reflections, transition to thick E-reflection band, and fully developed E-layer were successfully imaged. Dashed lines with identical colour-code and thickness show areas where imaging was intermittent. **b**, The location of the down-dip thermal limit for seismogenic behaviour, inferred to be about 350 °C from laboratory and field studies, has been estimated from numerical modelling across the Vancouver Island margin⁵. The landward limit of the locked zone has been estimated by matching the predicted vertical and horizontal deformation from elastic dislocation models for a locked fault, to observations of repeated levelling surveys, global positioning system and other geodetic data^{6,30}.

of the overlying upper mantle has been proposed for greater depths²⁰. The contact zone in this area of the thrust is probably quite thin, although there is some indication from the deep reflection profiles across the forearc of Chile that the broad reflection zone may continue below the forearc mantle wedge^{21,22}.

Our relocated seismicity, tomographic velocities, and reflection images (Fig. 3) indicate that the E-reflection zone lies just above the subducting oceanic slab. This identification is in agreement with some earlier interpretations^{9,10} although several other studies^{8,11,23} suggested that the E-reflection band is several kilometres above the subducting plate. Tomographic velocities from first-arrival data²⁴ (Fig. 3) only approximately outline the oceanic Moho, because of a 3-km cell size and a minimum structure assumption in the inversion. However, new 2D velocity models in Juan de Fuca Strait²⁵ were interpreted by inverting both refracted and reflected arrivals, including Moho reflections. These better constrain the oceanic Moho and infer that the top of oceanic crust is directly beneath the E-layer. The seismicity data suggest that a depth separation greater than about 2 to 3 km between the E-reflections and the oceanic plate cannot be accommodated, because relocated slab events occur within the top few kilometres below the E-layer

(Fig. 3). The E-reflection zone itself appears to be mostly aseismic. Only a small number of earthquakes occur within this zone at the landward end of line JDF1, and these may be explained by a few events being poorly located. It is thus likely that the E-layer represents the current slow slip zone.

In Fig. 4, we compare the variations in reflection character along the subduction thrust with the extent of the locked, transition and stable sliding zones determined from thermal and deformation studies: (1) The locked seismogenic zone correlates with the generally thin megathrust reflection package (zone 1). (2) The modelled transition zone correlates with the zone of gradual thickening of the E-zone reflections (zone 2). (3) The fault area that moved in the well-defined 1999 and other slow slip events⁶ correlates with the fully developed E-zone reflection package (zone 3). (4) The landward termination of the modelled 1999 slow slip area approximates the location of the abrupt termination of the E-zone reflections.

Our evidence suggests that the fully developed E-reflection zone demarcates the part of the subduction thrust where stable sliding and slow slip events dominate. Seaward thinning of the E-reflections delineates the transition zone towards the seismogenic part of the interplate interface that is characterized by narrow thrust reflections. The landward edge of the locked zone on the northern Cascadia subduction thrust inferred by reflection imaging appears to lie some 25–30 km closer to the land than estimated from thermal and dislocation modelling, implying a wider zone of coupling than currently proposed. If this wider zone is confirmed, a somewhat greater megathrust seismic hazard is suggested at inland cities. The transition zone based on reflection imaging is significantly narrower than the transition zone, as outlined by both thermal and dislocation modelling.

At the northern Cascadia subduction zone, deep reflection imaging has provided us with a method for detailed mapping of the expected rupture area of the subduction thrust, especially its landward limit, resulting in improved seismic hazard characterization. More accurate mapping of locked seismogenic zones requires calibration of the presented method at a subduction zone that has experienced megathrust earthquakes with the rupture extent defined by aftershocks and geodetic data. Deep seismic reflection images from Alaska²⁶, Chile²¹ and SW Japan²⁷ show a similar broad reflection band above the subduction thrust in the region of stable sliding and thin thrust reflections further seaward, perhaps suggesting that reflection imaging may be a globally important predictive tool for determining the maximum expected rupture area in megathrust earthquakes. □

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Catastrophic extinctions follow deforestation in Singapore

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The looming mass extinction of biodiversity in the humid tropics is a major concern for the future¹, yet most reports of extinctions in these regions are anecdotal or conjectural, with a scarcity of robust, broad-based empirical data^{2–4}. Here we report on local extinctions among a wide range of terrestrial and freshwater taxa from Singapore (540 km²) in relation to habitat loss exceeding 95% over 183 years^{5,6}. Substantial rates of documented and inferred extinctions were found, especially for forest specialists, with the greatest proportion of extinct taxa (34–87%) in

butterflies, fish, birds and mammals. Observed extinctions were generally fewer, but inferred losses often higher, in vascular plants, phasmids, decapods, amphibians and reptiles (5–80%). Forest reserves comprising only 0.25% of Singapore's area now harbour over 50% of the residual native biodiversity. Extrapolations of the observed and inferred local extinction data, using a calibrated species–area model^{7–9}, imply that the current unprecedented rate of habitat destruction in Southeast Asia¹⁰ will result in the loss of 13–42% of regional populations over the next century, at least half of which will represent global species extinctions.

Tropical forests represent the Earth's major reservoir of terrestrial biodiversity^{4,11}, yet these biomes are now gravely imperilled by anthropogenic change, including deforestation and habitat degradation^{12,13}, overexploitation of plant and animal populations¹⁴, and the introduction of invasive species¹. If we are to avert or at least mitigate catastrophic loss of species in these areas, it is vital to

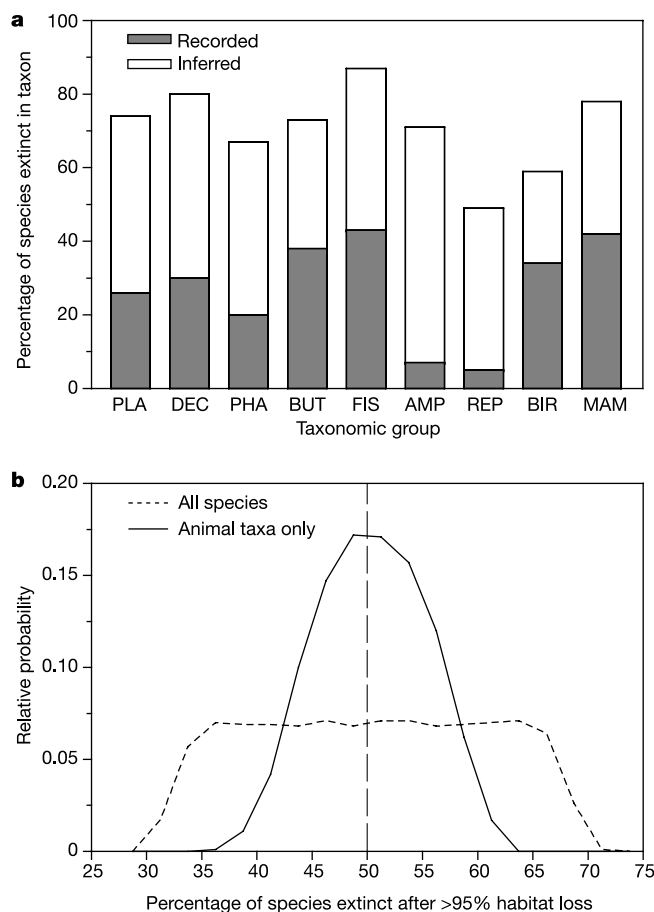


Figure 1 Observed and projected biodiversity loss in Singapore, 1819–2002.

a, Observed (recorded) and inferred extinction rates for vascular plants (PLA; number of modern, historically recorded and inferred species is 1,683, 2,277 and 6,549, respectively), freshwater decapod crustaceans (DEC; 16, 23, 82), phasmids (PHA; 33, 41, 100), butterflies (BUT; 236, 381, 863), freshwater fish (FIS; 35, 61, 269), amphibians (AMP; 25, 27, 87), reptiles (REP; 117, 123, 228), birds (BIR; 144, 218, 347) and mammals (MAM; 26, 45, 117). **b**, Combining the data from **a** provides a probability distribution of potential total species extinctions for all taxa (dashed line) and animal taxa only (continuous line), given a habitat loss exceeding 95% over a 183-year period. This was derived via computer re-sampling of the extinction data by assuming that the true extinction rate was equally and independently likely to lie anywhere between the observed and inferred proportions for each taxa. The vertical line indicates the mean of the animal taxa distribution.

understand the taxonomic and ecological selectivity¹⁵, rate⁸ and extent¹⁶ of local extinctions in already heavily deforested regions. However, until now a lack of appropriate knowledge and infrastructure in mostly developing tropical countries^{3,4,17} has forced an inevitable reliance on indirect measures and theoretical projections of extinctions^{2,7,9}.

We used both historical and modern checklists of species occurrences¹⁸ from tropical Singapore (103° 50' E, 1° 20' N) to estimate local extinctions¹⁹ in a wide range of taxa over a 183-year period in relation to large-scale habitat loss (Supplementary Information provides a complete catalogue of checklists). Since the British first established a presence in Singapore in 1819, more than 95% of the estimated 540 km² of original vegetation cover has been entirely cleared^{6,13}, and less than 10% of the 24 km² of remaining forest is primary (the majority being secondary re-growth)⁵. We focused on relatively well-studied taxonomic groups; that is, vascular plants, freshwater decapod crustaceans, phasmids, butterflies, freshwater fish, amphibians, reptiles, birds and mammals (excluding bats). However, given that a large fraction of the island's forest had already been cleared before the first reliable species records in the 1870s, the extinction of many species most sensitive to disturbance probably went unrecorded^{5,6,13,16}. To account for this gap, we also used checklists from nearby Peninsular Malaysia (assuming an equivalent species composition in corresponding habitats) to infer the possible pristine species composition of Singapore in 1819 (see Methods). The loss of local populations is highly relevant to global extinctions, because species disappear after all local populations have become extinct^{4,19}. Our analyses provide an empirical complement to the use of species distribution maps that infer less directly the extinction of populations from documented range reductions²⁰.

The observed local extinction rates in Singapore (Fig. 1a, shaded bars) were variable across taxonomic groups ($G = 67.4$, degrees of freedom (d.f.) = 8, $P < 0.001$). The overall loss of biodiversity was at least 28% (881 of 3,196 recorded species). Extinctions of butterflies, freshwater fish, birds and mammals (the most visible and well-studied animal taxa) were high (34–43%) and quite consistent ($G = 1.45$, d.f. = 3, $P = 0.694$) in the face of large-scale deforestation and habitat modification. Similarly, roughly a quarter of all vascular plant, freshwater decapod crustacean and phasmid species have disappeared. Conversely, amphibians and reptiles have apparently suffered comparatively fewer extinctions (5–7%). Our estimates of the possible (inferred) biodiversity loss in Singapore (Fig. 1a, white bars) suggest that the total local extinction of species (recorded and unrecorded) may be as high as 73%. The differences between observed and inferred extinctions are relatively small for some taxa (for example, 34–59% in birds), but substantial in others (7–71% in amphibians). A simple combinatorial simu-

lation of the observed–inferred proportional extinction bounds data (see Methods) produces a probability distribution of total biodiversity loss (Fig. 1b) peaking at 50.4% (95% confidence interval = 41.2–59.6%).

Most evidence indicates that the true biodiversity loss in Singapore is closer to our inferred than observed estimates. Although extinctions are notoriously difficult to document with certainty²¹, the small and intensively sampled area of remaining forest means that our extinction data probably include few 'false negatives'. Our extant species lists also include long-lived species with populations too small to be viable in the long term (for example, white-bellied woodpecker *Dryocopus javensis* (<5 individuals present), banded leaf monkey *Presbytis femoralis* (<15) and cream-coloured giant squirrel *Ratula affinis* (<10))²². These 'living dead' taxa will almost certainly become extinct in the coming decades^{4,8}. Some buffering of Singapore's local populations through re-colonization from neighbouring Malaysia has probably occurred (thereby increasing their resilience to extinction), but is believed to have been relatively low because of the poor dispersal abilities of most of the taxa treated, as well as ethological and habitat barriers (water, extensive mangroves and urban and agricultural development along the adjacent coastlines) impeding even mobile species such as birds²³. Peninsular Malaysia itself has already lost about 60% of its pristine habitat in historical times (so past unrecorded extinctions are also expected there)⁴, and its biota has not been as exhaustively studied as Singapore's. This suggests that Peninsular Malaysia has almost certainly experienced some extinctions in the past 200 years, and that it is unlikely the current biodiversity of this region substantially exceeds the past diversity in Singapore, despite the latter's smaller geographical area. We therefore regard our inferred extinction estimates as conservative.

The differing magnitude of observed extinctions across taxonomic groups is probably due in part to a complex generational scaling effect on long-term population persistence. Larger organisms require more habitat area to support viable populations, but also tend to live longer²⁴. These taxa (including most remaining tree species) may persist long after habitat reduction²⁵, but carry the burden of a past habitat-loss debt²⁶ that consigns them to future extinction^{4,6}. Invertebrates (for example, butterflies) require relatively less habitat to persist, but have already passed through many generations of 'relaxation'⁸ since deforestation, and may therefore most closely reflect the equilibrium end-point for all taxa. Small vertebrates (for example, frogs and lizards) also need less pristine forest area than birds or mammals, but have longer generation times than invertebrates²⁴ and have therefore spent fewer generations near a critical extinction threshold. It is also likely that the existence and subsequent extinction of many smaller, more cryptic organisms simply went unrecorded. For example, four of the six species of

Table 1 Potential future biodiversity losses in Singapore and extrapolations to Southeast Asia

Taxon	Percentage of species restricted to Singapore's reserves*	Total percentage extinct with loss of reserves†	Projected percentage biodiversity loss in SE Asia by 2100‡
Vascular plants	Majority of native species ^{6,25} (not quantified)		12–44
Decapods [§]	81 (13)	87 (× 2.9)	14–50
Phasmids	100 (33)	100 (× 5.1)	9–38
Butterflies	63 (149)	77 (× 2.0)	19–43
Fish [§]	60 (21)	77 (× 1.8)	21–58
Amphibians	76 (19)	78 (× 10.5)	3–41
Reptiles	50 (59)	52 (× 10.7)	2–25
Birds	8 (12)	39 (× 1.1)	16–32
Mammals	46 (12)	69 (× 1.6)	21–48
Weighted Mean	50 (312)	66 (× 2.1)	13–42

*Reserves are Bukit Timah (71 ha), Nee Soon (935 ha), MacRitchie (484 ha), Lower Pierce forest (50 ha) and Botanic Gardens (7 ha). Numbers in parentheses are total number of species per taxon restricted to the reserves.

†Calculated as (number species extinct + number species restricted to reserves)/original number of species. Numbers in parentheses indicate (number species extinct + number species restricted to reserves)/number species extinct; for example, (×2.0) signifies a twofold increase in the extinction rate. Based on recorded extinctions only, so represents a minimum estimate of biodiversity loss.

‡Extrapolation of regional population extinctions based on a species–area curve scaling constant z fitted to each taxon's respective recorded and inferred habitat loss–extinction rate relationship in Singapore, proportion of original regional forest cover lost to date, and the present rate of deforestation in Southeast Asia as documented by ref. 10.

§Freshwater only (excludes marine species).

freshwater crabs on the island were discovered only over the last 20 years²².

Most extinctions occurred among those biota restricted to forest habitats (primary or secondary rainforest or mangrove forest interiors). The overall documented local extinction rate for forest specialists was 33% ($n = 2,542$), compared with only 7% ($n = 654$) for species that prefer or tolerate open or forest-edge habitats. These habitat-specific extinctions were particularly severe in freshwater fish, birds and mammals, which lost 53%, 67% and 59% of their forest species, respectively, compared with only 0%, 11% and 13% of their open-habitat counterparts.

Forest birds provide the best quantitative data on the rate and selectivity of the extinctions. Singapore's contemporary bird fauna is characterized by a predominance of open-habitat generalists, migrants and invasives²⁷, because since 1923, 61 of the 91 known forest-dependent species have become extinct¹⁶. Furthermore, up to 168 species may have once resided in Singapore (based on forest bird diversity in Peninsular Malaysia), implying extinction of up to 46% of species before the first reliable checklists were compiled (in strong agreement with other measures of relaxation rates in tropical bird communities⁸), and a total species loss of 82% since 1819. A binary logistic regression model relating the likelihood of extinction to habitat preference (forest interior, forest edge, open) and body length (mm) was highly significant ($G = 94.5$, d.f. = 4, $P < 0.001$) and provided strong concordance with the empirical extinction data (76.3% overall concordance, or 65.9% for habitat alone). Large forest-interior species were most vulnerable and small open-habitat-preferring species least so.

The predominant cause of Singapore's extinctions was undoubtedly rapid and large-scale habitat destruction, initially through deforestation for agriculture, and later, urban development⁵. Habitat loss, fragmentation and modification cause extinctions by reducing breeding and feeding sites, increasing predation, soil erosion and nutrient loss, limiting dispersal, and enhancing edge effects^{1,4,12,26}. However, factors such as over-hunting and collecting may have also been important^{4,14}, especially for the larger vertebrates—the last tiger (*Panthera tigris corbetti*) was shot in 1930. In addition, heavy shelling of the nature reserves during World War II had an undocumented but presumably detrimental impact on the forest fauna¹⁶.

The future prospects for Singapore's surviving biodiversity look bleak—77% of the island's species are considered 'threatened', based on the World Conservation Union (IUCN) regional listing criteria²². Furthermore, our estimates show that the few remaining protected nature reserves, which occupy only 1,547 ha (or 0.25% of the total land area)^{5,16}, harbour exclusively at least 50% (316) of species from the animal taxa we assessed, and a high (but unquantified) number of plant species. The loss of these reserves would more than double the observed fraction of species driven to extinction (Table 1). Birds are unusual among the taxonomic groups we examined in having few species dependent on the reserves, but only because most of the forest species are already extinct¹⁶. Of particular concern is the lack of redundancy in the present reserve system. For instance, a quarter of the surviving freshwater decapod crustacean and fish fauna are now restricted to only a small (5 ha) area in a single reserve (Nee Soon)²⁸, reinforcing the view that reserve designs that emphasise single representations of many species can be a poisoned chalice²⁹.

What can local extinctions in Singapore tell us about the larger biodiversity crisis now affecting the tropical forests of Southeast Asia^{4,7,12}, which support a comparable biota? Few other areas in Southeast Asia have hitherto suffered from such extensive habitat loss as Singapore (regional average is 46% loss of original forest cover⁷), yet projections of current rates of regional deforestation ($0.71\% \text{ yr}^{-1}$)¹⁰ imply an overall deforestation of 74% in Southeast Asia by the year 2100. We fitted our observed and inferred extinction data to the species–area relationship^{7–9,16}, and used this empirically

calibrated model to estimate the number of species likely to become extinct or consigned to extinction in Southeast Asia over the next century (see Methods). We predict the overall loss of 13–42% of regional populations due to the effects of deforestation in Southeast Asia by the end of the present century (see Table 1 for a taxonomic break down), at least half of which are likely to represent global species extinctions, given estimates of regional endemism^{3,7,11}. Realized extinctions may be even higher, given the additional pressures of overexploitation, displacement by exotic species, climate change and loss of non-forest habitats^{1,4,14}, proportionally higher rates of deforestation in the biodiversity hotspots^{10,11}, and probable buffering of local populations in Singapore by recolonizations. Clearly, large-scale conservation efforts need to be implemented if these regional rates of extinction are to be abated. □

Methods

Quantifying local extinctions

The equatorial island of Singapore offers a unique opportunity to analyse the long-term phenomenological response of tropical biota to massive habitat loss, fragmentation and degradation, providing a 'microcosm' of the tropical biodiversity crisis. Its typically Southeast Asian terrestrial and freshwater biotas have been studied in detail by amateur naturalists and professional biologists for over a century^{5,16,30}, and modern biodiversity surveys have provided a relatively exhaustive coverage of the few remaining habitat areas²². Past and present taxonomic inventories from Singapore were compiled by sourcing all primary, secondary and 'grey' literature (checklists and scientific surveys) and the major contemporary biological reference material covering Peninsular Malaysia (see the Supplementary Information for a full listing). Recorded extinctions were defined using standard IUCN guidelines^{6,22}. Observed proportional extinctions were calculated as $(H - M)/H$ and inferred extinctions as $(P - M)/P$, where H and M are the size of the historical and modern Singaporean checklists, respectively, and P is the size of the modern checklists of Peninsular Malaysia.

We excluded montane, savanna, lacustrine and large river taxa from the species list for Peninsular Malaysia because Singapore lacks these habitats. Singapore has been separated from Peninsular Malaysia by a narrow (600-m wide), shallow (10-m deep) strait for only a few thousand years⁵, resulting in very low endemism, with only three endemic decapod crustacean species and one endemic subspecies of bird from the taxa studied. Therefore, the extinctions that we are reporting are population extinctions^{19,20}. These compiled data show that Singapore possessed a minimum (if we consider only documented records) of 37% of the biodiversity of Peninsular Malaysia (63% for birds).

Simulation of the uncertainty distribution of extinctions

Our estimates of the observed and inferred total species losses were combined by assuming: (1) that the true point estimate of the fraction of extinct biota for each taxon falls somewhere between these two bounds, with equal probability (that is, a uniform distribution); and (2) that the position of the extinction fraction within these bounds was independent across taxa. Repeated computer-aided re-sampling of point estimates within the extinction bounds data for each taxonomic group was combined across all represented taxa (and separately, just the animal taxa) and used to construct a probability–frequency distribution of species loss (Fig. 1b, based on 30,000 simulation replicates). Vascular plants comprised 71–77% of Singapore's total original biodiversity of these groups, and thus had a strong influence on the shape and uncertainty of the resulting probability distribution.

Extrapolation to Southeast Asia

Previous estimates of possible future biodiversity loss in Southeast Asia^{7,15,16} have relied on extrapolations using the species–area relationship (SAR), defined as $S = cA^z$, where S is the ratio of contemporary to original species composition, A is the ratio of present to original habitat area, and c and z are constants^{8,9}. Previous extrapolations have assumed a value of $z = 0.25$. We built on this approach by using our empirical data to 'calibrate' z for each of the nine taxonomic groups we studied, and used both the observed and inferred local extinction estimates to encompass the major uncertainties.

To solve the SAR for z , we used a different S for each taxonomic group and set A for Singapore as $4.3\%^{5,6,13}$, resulting in values of z and S (observed, inferred) as follows: vascular plants, $z = (0.10, 0.43)$ and $S = (0.74, 0.26)$; freshwater decapod crustaceans, $z = (0.11, 0.51)$ and $S = (0.70, 0.20)$; phasmids, $z = (0.07, 0.35)$ and $S = (0.80, 0.33)$; butterflies, $z = (0.15, 0.42)$ and $S = (0.62, 0.27)$; freshwater fish, $z = (0.18, 0.65)$ and $S = (0.57, 0.13)$; amphibians, $z = (0.02, 0.40)$ and $S = (0.93, 0.29)$; reptiles, $z = (0.02, 0.22)$ and $S = (0.95, 0.51)$; birds, $z = (0.13, 0.28)$ and $S = (0.66, 0.41)$; mammals, $z = (0.17, 0.48)$ and $S = (0.58, 0.22)$; weighted average, $z = (0.11, 0.42)$ and $S = (0.73, 0.28)$. Extrapolations for Southeast Asia from 1997 to 2100 (a 103-year period) assumed an annual regional deforestation rate¹⁰ of $0.71\% \text{ yr}^{-1}$ and $A_{(1997)} = 46\%$, giving $A_{(2100)} = 0.54(1 - 0.0071)^{103}$.

Our approach has the advantage of incorporating regional- and taxon-specific information into the projections (than rather than assuming a single universal value of z for all groups), and emphasises the complementary way in which local extinction and regional habitat-loss studies can be combined to produce biologically and empirically

grounded estimates of future biodiversity loss. Limitations on the reliability of this method include the difficulty in estimating the proportion of contemporary biota in Singapore that has not yet reached relaxation (that is, presently surviving, but committed to future extinction)^{13,25}, the unknown extent to which supplemental migration from mainland Malaysia may have buffered local populations in Singapore from extinction, the uncertainties in estimating the true pristine biodiversity of the island, given the likelihood of past habitat-loss-related extinctions in Peninsular Malaysia, and the uneven geographical distribution of endemic biodiversity 'hotspots'²¹, which currently suffer from higher rates of deforestation and degradation than the average of the entire region¹⁰. Collectively, these potential biases suggest that our projected regional losses are likely to be conservative.

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Productivity–biodiversity relationships depend on the history of community assembly

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Identification of the causes of productivity–species diversity relationships remains a central topic of ecological research^{1,2}. Different relations have been attributed to the influence of disturbance^{3,4}, consumers^{5,6}, niche specialization⁷ and spatial scale^{8–14}. One unexplored cause is the history of community assembly, the partly stochastic sequential arrival of species from a regional pool of potential community members. The sequence of species arrival can greatly affect community structure^{15–19}. If assembly sequence interacts with productivity to influence diversity, different sequences can contribute to variation in productivity–diversity relationships. Here we report a test of this hypothesis by assembling aquatic microbial communities at five productivity levels using four assembly sequences. About 30 generations after assembly, productivity–diversity relationships took various forms, including a positive, a hump-shaped, a U-shaped and a non-significant pattern, depending on assembly sequence. This variation resulted from idiosyncratic joint effects of assembly sequence, productivity and species identity on species abundances. We suggest that the history of community assembly should be added to the growing list of factors that influence productivity–biodiversity patterns.

Productivity, the amount of energy available for ecosystem development in a given location, has a major effect on species diversity^{20–22}. Until recently, hump-shaped relationships, in which diversity peaks at intermediate productivity levels, were the most widely observed pattern^{23–25}. We now know that the relationship takes many forms, including hump-shaped, U-shaped, positive, negative and flat (non-significant) patterns, and that none of these patterns predominates¹. Possible causes of variation include the influence of disturbance^{3,4}, consumers^{5,6}, niche specialization⁷ and spatial scale^{8–14}, which can create variation between taxonomic groups and habitat types^{1,13}. A few studies suggest that productivity might control the probability that alternative community states are produced through assembly^{14,26–28}.

Table 1 Introduction sequences used to assemble communities

	Sequence			
	A	B	C	D
First introduction	Set 1	Set 1	Set 2	Set 2
Second introduction	Set 2	Set 3	Set 1	Set 3
Third introduction	Set 3	Set 2	Set 3	Set 1
Set 1	Set 2	Set 3		
<i>Blepharisma americanum</i> *†	<i>Colpidium striatum</i> *	<i>Aspidisca</i> sp.*		
<i>Chilomonas</i> sp.*	<i>Colpoda cucullus</i> *	<i>Holosticha</i> sp.*		
<i>Colpoda inflata</i> *	<i>Euplotes</i> sp.*†‡	<i>Lepadella</i> sp.* (r)		
<i>Loxoxcephalus</i> sp.*	<i>Paramecium tetraurella</i> *†	<i>Rotaria</i> sp.* (r)		
<i>Paramecium caudatum</i> *†	<i>Tetrahymena vorax</i> *†	<i>Spirostomum</i> sp.*		
<i>Tetrahymena thermophila</i> *	<i>Uronema</i> sp.*	<i>Tillina magna</i> *		

The natural history of these rotifers (marked with (r)) and protozoans (all others) indicates that they consume bacteria and/or microflagellates (*), algae (†), and/or small ciliates (‡). Regardless of their diets, all the species can sustain their population solely on bacteria and/or microflagellates and thus potentially compete with one another.

Assembly sequence:

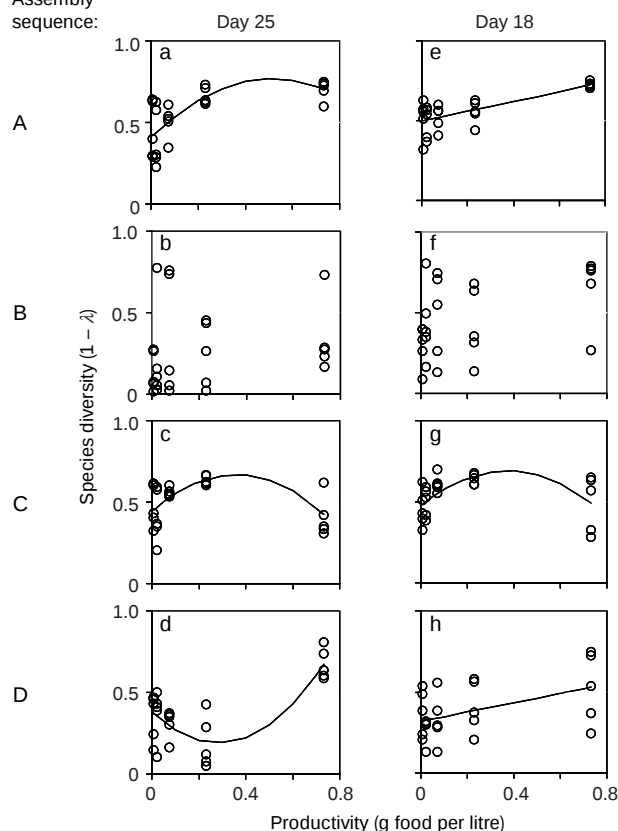


Figure 1 Response of species diversity to productivity. Data are fitted to the best regression models. Productivity refers to protozoan pellet concentration. Some data points are slightly moved vertically (no greater than ± 0.005) from their original points so that they can be distinguished more clearly from one another.

We conducted a laboratory experiment using freshwater microbial communities as a model system to test for interactive effects of assembly and productivity on diversity. We manipulated productivity by changing the nutrient concentration of the medium. At each of the five productivity levels used, we first inoculated the medium with bacteria, microflagellates and algae. After allowing them to become abundant, we assembled communities by using four different introduction sequences of 18 protozoan and rotifer species, which consumed bacteria, microflagellates and algae, and thus potentially competed for these shared resources (Table 1). We used a total of 100 microcosms, namely 5 productivity levels $\times$ 4 assembly sequences $\times$ 5 replicates for each treatment.

Species diversity was expressed as the complement of Simpson's index²⁹, $1 - \lambda = 1 - \sum p_i(1 - p_i)$, where p_i is the relative frequency of species i , using data obtained 18 and 25 days after the last introduction. We examined the relationship between productivity and species diversity by fitting data to the following models:

$$\text{model 1: } d = b_0 + b_1 p$$

$$\text{model 2: } d = b_0 + b_1 p + b_2 p^2$$

$$\text{model 3: } d = b_0 + b_1 \log_{10} p$$

$$\text{model 4: } d = b_0 + b_1 \log_{10} p + b_2 (\log_{10} p)^2$$

where d is species diversity ($1 - \lambda$), p is productivity (grams protozoan pellet per litre), and b_0 , b_1 and b_2 are regression parameters. Models 1 and 3 linearly relate species diversity to productivity and to log-transformed productivity, respectively. A quadratic term is added to models 1 and 3 to form models 2 and 4, respectively, to test for curvilinearity.

The use of microcosms permitted rigorous control over assembly history, productivity and other environmental conditions. It also

Table 2 Response of species to productivity and assembly sequence

Species	Productivity				
	1 (highest)	2	3	4	5 (lowest)
<i>Aspidisca</i> sp.	<u>D C B A</u> (2.89)	<u>B D C A</u> (1.98)	<u>D B C A</u> (1.82)	<u>C B A D</u> (0.88)	<u>C A B D</u> (9.22**)
<i>Blepharisma americanum</i>	<u>A B D C</u> (13.83***)	<u>A C D B</u> (1.69)	<u>C D A B</u> (0.67)	<u>A D C B</u> (3.30)	<u>A D C B</u> (6.65**)
<i>Chilomonas</i> sp.	<u>D C A B</u> (37.54***)	<u>D A C B</u> (1.00)	<u>D A C B</u> (1.00)	<u>D A C B</u> (1.00)	—
<i>Colpoda cucullus</i>	<u>A B C D</u> (1.00)	<u>A B C D</u> (0.83)	<u>A B C D</u> (1.92)	<u>A B D C</u> (2.63)	<u>A D C B</u> (5.92**)
<i>Euplates</i> sp.	<u>A C B D</u> (12.13***)	<u>C A B D</u> (6.78**)	<u>C D B A</u> (4.21*)	<u>C A B D</u> (19.50***)	<u>C A B D</u> (5.11*)
<i>Holosticha</i> sp.	<u>C A D B</u> (3.86)	<u>C A D B</u> (24.19***)	<u>A B D C</u> (4.07)	<u>D B C A</u> (1.09)	<u>B D C A</u> (0.28)
<i>Lepadella</i> sp.	<u>A B C D</u> (4.98)	<u>B A D C</u> (7.42**)	<u>B D C A</u> (2.20)	<u>A B D C</u> (0.86)	<u>D C A B</u> (1.79)
<i>Paramecium tetraurelia</i>	<u>C D B A</u> (7.75**)	<u>B A D C</u> (4.25)	<u>A D C B</u> (0.42)	<u>A C D B</u> (0.82)	<u>A B D C</u> (3.58)
<i>Rotaria</i> sp.	<u>B A C D</u> (160.78***)	<u>B A D C</u> (11.13***)	<u>B A D C</u> (13.21***)	<u>B D A C</u> (6.98**)	<u>D B A C</u> (2.96)
<i>Spirostomum</i> sp.	<u>C A B D</u> (1.03)	<u>C A B D</u> (1.57)	—	—	—
<i>Uronema</i> sp.	<u>C B D A</u> (16.69***)	<u>D B A C</u> (12.90**)	<u>B D A C</u> (0.33)	<u>B D A C</u> (2.37)	<u>B D C A</u> (8.04**)

For each species and productivity level, assembly sequences (A, B, C and D) are listed in order of decreasing abundance on day 25. Underlined groups cannot be distinguished statistically with Tukey's studentized range tests. Numbers in parentheses indicate F -ratio (ANOVA). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ (only those found to be significant after a sequential Bonferroni correction to preserve a Type 1 error rate of 0.05 are asterisked). Dashes indicate that the species went extinct in all replicates at the corresponding productivity level. Species not listed here went extinct in all replicates at all productivity levels.

enabled us to observe patterns resulting from long-term community dynamics. Use of an experimental duration spanning tens of generations limited the possibility that observed patterns were trivial over ecologically important timescales. It would have been impossible to ensure this level of experimental control in most other natural or laboratory settings. Because all of our microcosms ultimately received the same set of species, any differences in productivity–diversity relations could be attributed to different assembly sequences.

The specific assembly sequence used to create communities generated striking differences in productivity–diversity relationships. Statistical analysis (see Methods) revealed positive ($F = 11.23$, $P = 0.0004$, adjusted $R^2 = 0.4601$ for the best model (model 2); Fig. 1a), non-significant (flat; $F < 1.45$, $P > 0.2425$, adjusted $R^2 < 0.0181$ for all the models; Fig. 1b), hump-shaped ($F = 6.31$, $P = 0.0068$, adjusted $R^2 = 0.3066$ for the best model (model 2) and $P < 0.05$ for Mitchell-Olds & Shaw's test³⁰; Fig. 1c) and U-shaped ($F = 19.96$, $P < 0.0001$, adjusted $R^2 = 0.124$ for the best model (model 2) and $P < 0.05$ for Mitchell-Olds & Shaw's test³⁰; Fig. 1d) patterns under sequences A, B, C and D, respectively, 25 days after introduction of the last species (see also Supplementary Information). This endpoint corresponded to about 30 complete generations of the organisms involved in community dynamics.

These results also qualitatively hold for data collected 18 days after the last introduction (Fig. 1e–h), confirming that the patterns were temporally consistent and long-lived over ecologically important timescales (see also population dynamics in Supplementary Information). On both days, the relationship was positive, flat (non-significant) and hump-shaped under sequences A, B and C, respectively. Under sequence D, the best model was positive linear for day 18 (Fig. 1h), whereas it was U-shaped for day 25 (Fig. 1d). However, model selection was relatively indecisive for this sequence on day 18, and a U-shaped model (adjusted $R^2 = 0.181$, model 4) explained data almost as well as the positive linear model selected did (adjusted $R^2 = 0.184$, model 1).

We examined how each species responded to productivity and

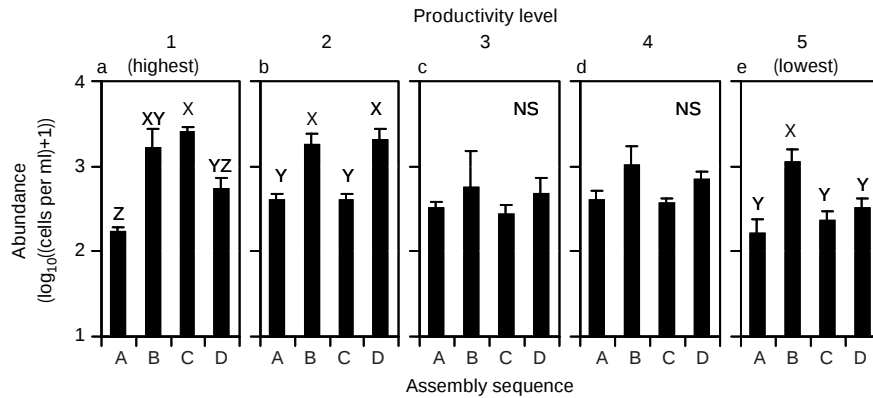


Figure 2 Response of the abundance of *Uronema* sp. to productivity and assembly sequence on day 25. Bars with the same letter above them did not differ in Tukey's

studentized range tests. We applied a sequential Bonferroni correction to preserve a Type I error rate of 0.05. NS, not significant (see Table 2). Error bars are s.e.m.

assembly sequence to search for processes that might have created different patterns. For many species, the effect of assembly sequence on abundance depended on productivity. For example, the assembly sequence significantly influenced the abundance of *Uronema* sp. at three out of five productivity levels (Fig. 2). When significant, the effect of assembly sequence on abundance also depended on productivity. *Uronema* was more abundant in sequence C than in sequence D at one productivity level (Fig. 2a), but the difference was reversed at another level (Fig. 2b) and became non-significant at the other levels (Fig. 2c–e). Thus, productivity determined whether a certain sequence either facilitated or inhibited population growth. Furthermore, the effect of sequence also depended on species identity (Table 2). Joint effects of assembly sequence, productivity and species identity were highly idiosyncratic, with no discernible general trends across species (Table 2).

Our results show that community assembly potentially interacts with productivity to create a remarkable variety of productivity–diversity patterns. We emphasize that different assembly sequences alone produced various productivity–diversity patterns, without experimental imposition of variation in disturbance^{3,4}, consumers^{5,6}, niche specialization⁷ or spatial scale^{8–14}. We suspect that assembly sequence might have influenced the operation of these unmanipulated proximate mechanisms¹⁷. For example, some sequences might have caused consumers and niche specialization, realized by diet differences between species (Table 1), to exert a strong effect on productivity–diversity patterns, whereas other sequences might have minimized their influence. The next step in understanding this phenomenon would be to uncover general mechanisms that explain how assembly produces such diverse patterns. Our results caution against assuming that a single explanatory mechanism at the community level exists, because interactions between species apparently depend in a complex way on assembly sequence, productivity and species identity (Table 2).

Our interpretation of these data assumes that the spatial scale of productivity variation is smaller than that for assembly sequences. Some natural systems meet this assumption. Examples include situations where regular seasonal phenology of species arrival drives assembly or where species invade a region through biogeographic events and spread before the next species invade. Other systems contain local sites that vary in both productivity and assembly sequence. However, we note that assembly has important implications even for these systems: it can make patterns sensitive to the scale of observation¹⁴. We found that the productivity–diversity relationship was positive linear at a local scale ($P = 0.0017$ and adjusted $R^2 = 0.3255$ for model 1, which was the best model), whereas it was non-significant at a regional scale ($P > 0.02$ for all models; note that $\alpha = 0.0125$ with Bonferroni correction; see

Supplementary Information for how we calculated local and regional diversity).

We studied only four of the many possible assembly sequences that could have been used with our species pool. This small sample of assembly sequences makes it impossible to say whether any one of the specific patterns that we observed might predominate in a larger sample. Nevertheless, it is possible that the variety of productivity–biodiversity relationships seen in nature reflects differences in assembly as well as other factors. We suggest that the history of community assembly should be added to the growing list of factors that influence productivity–biodiversity patterns. Assembly history does not preclude the importance of other factors. However, because the detailed assembly history of natural communities is seldom known, it might be difficult to deduce the proximal causes of natural productivity–diversity patterns unambiguously. For this reason, manipulative experiments remain an essential tool for exploring the possible causes of productivity–diversity relationships within the historical context of community assembly. □

Methods

Microcosms

Microcosms were covered sterile 118-ml polypropylene containers. These containers were filled with 30 ml of medium (made from Carolina Biological Supply protozoan pellet, Herpetovite powdered vitamin supplement, and soil in well water) and kept at 22 °C with 14 h light/10 h dark cycles. The medium was autoclaved before use and inoculated with bacteria (*Bacillus subtilis*, *Bacillus cereus*, *Proteus vulgaris*, *Serratia marcescens* and other unidentified bacteria filtered from the stock cultures of all the protozoan and rotifer species used in the experiment), microflagellates and algae (*Chlamydomonas* spp.). These inoculations were done before the medium was distributed to microcosms.

Manipulating productivity

We used five levels of productivity, evenly spaced on a logarithmic scale. The medium for the lowest productivity level consisted of 0.007 g of the protozoan pellet, 0.033 g of the soil and 0.001 g of the vitamins in each litre of well water. The media for the other productivity levels consisted of 0.023, 0.073, 0.232 and 0.733 g of the pellet, 0.106, 0.334, 1.055 and 3.335 g of the soil, and 0.004, 0.013, 0.042 and 0.133 g of the vitamins per litre, respectively, from the second lowest to the highest levels. Removal and replacement of 3 ml (that is, 10%) of the medium of the corresponding nutrient concentration once a week renewed nutrients.

Manipulating assembly sequence

Protozoan and rotifer species were introduced to the microcosms sequentially in accordance with predetermined schedules (Table 1). Stock cultures of six species were used for each introduction. First species were introduced 3 days after the algal inoculation. Subsequent introductions had 14-day intervals. Microcosms received a very small number of individuals, namely less than 0.7% of carrying capacity, but at least 20 individuals per species to preclude trivial extinction by chance at initial stages. To standardize the number of individuals introduced across introduction occasions, population densities in stock cultures were estimated and, if necessary, diluted before introductions. The age of the stock cultures at the time of species introductions was also standardized to minimize variation in physiological conditions of species between different introduction occasions. Microcosms also contained three additional algal species. Because these algae were most probably from cultures of a particular species (*Euplotes* sp.), they did not confound the effect of assembly sequence.

Measuring population abundances

The population abundance of each species in each microcosm was measured once a week until 25 days after the last introduction. Twenty-five days corresponds to roughly 30 generations of the protozoa and rotifer species. Densities were estimated by counting protozoa and rotifers in samples of known volume, typically 0.3 ml, from the 3 ml of medium removed for nutrient replacement. When species were too abundant to count reliably, the sample was diluted. When one or more species were absent from the 0.3-ml sample, the entire 3 ml of medium (and the entire microcosm on the last sampling occasion) was scanned and protozoa and rotifers were counted.

Productivity–diversity relationships

When $P > 0.0125$ (that is, 0.05/4, using a Bonferroni correction to retain a Type I error rate of 0.05) for all models (see model description in text), we concluded that the relationship between productivity and diversity was not significant. When $P < 0.0125$ for more than one model, we selected as the best model the one that had the highest value of adjusted R^2 . Adjusted R^2 values are adjusted for the number of parameters in the models. When model 2 or 4 was selected, we determined whether the relationship was hump-shaped or U-shaped by using Mitchell-Olds & Shaw's test³⁰ (see Supplementary Information). We focused on the diversity of protozoan and rotifer species; our diversity index does not include bacteria, microflagellates or algae. Productivity–diversity relationships depended on assembly history when species richness (number of species per unit volume), rather than the complement of Simpson's index, was also used to express species diversity (see Supplementary Information).

Response of species

We conducted analyses of variance to test for effects of introduction sequence on species abundance at each productivity level. Abundance was transformed as $\log_{10}((\text{individuals per ml}) + 1)$ before analysis to minimize heteroscedasticity. For each species we used a sequential Bonferroni correction for the five tests corresponding to the five productivity levels to preserve a Type I error rate of 0.05. When analyses of variance found a significant effect of sequence, Tukey's studentized range tests were used to identify which treatments differed.

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The role of neuronal identity in synaptic competition

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In developing mammalian muscle, axon branches of several motor neurons co-innervate the same muscle fibre. Competition among them results in the strengthening of one and the withdrawal of the rest^{1,2}. It is not known why one particular axon branch survives or why some competitions resolve sooner than others³. Here we show that the fate of axonal branches is strictly related to the identity of the axons with which they compete. When two neurons co-innervate multiple target cells, the losing axon branches in each contest belong to the same neuron and are at nearly the same stage of withdrawal. The axonal arbor of one neuron engages in multiple sets of competitions simultaneously. Each set proceeds at a different rate and heads towards a common outcome based on the identity of the competitor. Competitive vigour at each of these sets of local competitions depends on a globally distributed resource: neurons with larger arborizations are at a competitive disadvantage when confronting neurons with smaller arborizations. An accompanying paper tests the idea that the amount of neurotransmitter released is this global resource⁴.

A central feature of mammalian neural development is the reapportionment of synaptic contacts such that neurons progressively innervate fewer postsynaptic cells but with more synapses^{5–7}. Synapse elimination at the skeletal neuromuscular junction is currently the best studied of all such rearrangements and viewed by some as a model for changes that occur in the developing brain. In the neuromuscular system of neonatal rodents, the number of muscle fibres contacted by one motor neuron decreases during the first two postnatal weeks until each muscle fibre is innervated by only one axon⁸. Within the arbor of a single motor axon, this branch withdrawal is protracted and asynchronous; after some terminal branches have definitively won or lost at some neuromuscular junctions, other branches of the same neuron still share synaptic sites with other innervating axons³. Several lines of evidence suggest that competitions between axon branches underlie this process^{9,10}. It is not known, however, what properties of an axonal branch or its environment determine its destiny in these competitions. Here, we ask whether the competitive vigour of each axon branch is determined by local factors or rather is set by a global property of the parent neuron. If axonal branches were acting as agents of their

parent neurons, then synapse withdrawal should be influenced by the neuronal identities of competing axonal branches. To test this idea, we asked the following question (shown diagrammatically in Fig. 1a): when the same two axons co-innervate multiple post-synaptic cells, is the outcome skewed in favour of the same axon at all common targets?

We bred together transgenic mice that expressed either yellow or cyan fluorescent proteins (YFP or CFP) in a small, random subset of motor neurons¹¹ to create double-subset-expressing mice (see Methods). We screened approximately 200 mice between postnatal day seven (P7) and P9, to find seven limb muscles containing both a CFP- and YFP-expressing axon that co-innervated multiple neuromuscular junctions (muscles I–VII, Fig. 2). High-resolution confocal reconstructions (Fig. 1b) of the axonal branching pattern and the postsynaptic sites containing acetylcholine receptors were combined into a large montage that provided both the complete branching pattern of each motor axon and the deployment of each axon's terminals at the co-innervated junctions (Fig. 1c).

In the example shown in Fig. 1c, the CFP- and YFP-labelled axons ramified over nearly the same large portion of this muscle's multiple endplate bands and co-innervated ~1% (that is, 12) of the muscle fibres in this muscle (I, Fig. 2; see also Fig. 1c). Given the ages examined in this study (P7–P9), most of these multiply innervated muscle fibres were likely contacted by only the two labelled axons⁹. There was no suggestion either in terms of location in the muscle (Fig. 1c) or location within the branching tree of each axon (Supplementary Fig. 1a, b) that these axon branches or the muscle

fibres they co-innervated were exceptional.

However, by two separate criteria, the innervation of the co-innervated junctions was dramatically biased in favour of the CFP axon (Fig. 3a–h). First, the YFP axon occupied on average $15 \pm 4\%$ ($n = 12$) of the acetylcholine receptor (AChR) territory of the co-innervated neuromuscular junctions, whereas all of the CFP-expressing branches occupied more than 50% (average CFP occupation $76 \pm 3\%$, $P < 0.0001$, Mann–Whitney test, Fig. 3i; column a, Fig. 2), a 5.7 ± 1.1 -fold difference in average area of occupation. Second, the mean YFP axon calibre was about half the calibre of CFP axon branches innervating the same junctions ($0.55 \pm 0.11 \mu\text{m}$ versus $1.2 \pm 0.15 \mu\text{m}$, $n = 12$, $P < 0.03$, Mann–Whitney test, Fig. 3j). Axons that withdraw from sites of synaptic competition become thinner than their competitor³. These results suggest that at each co-innervated junction, competition favoured the CFP axon.

Analysis of the entire set of terminal branches of these two motor axons (that is, their motor units) showed that both had a similar wide range of occupied territories and axon calibres (Fig. 3k–n), ruling out the possibility that the YFP axon generally occupied smaller synaptic territories and its branches had smaller axon calibres than the CFP axon. In addition, the junctions co-innervated by the two axons were equally likely to be contacted by a more proximal branch of the CFP axon as contacted by a more proximal branch of the YFP axon (Supplementary Fig. 1c), ruling out a systematic difference between the branch order of the two axons that co-innervated common junctions. Finally, a Monte Carlo analysis¹² of the sizes and areas of all 120 branches of the YFP axon showed that, within the YFP motor unit itself, the skewing towards smaller synaptic territories ($P < 0.006$, column b, Fig. 2) and calibres ($P < 0.02$) at the cohort of junctions co-innervated by the CFP axon was highly unlikely to be a chance event.

Similar results were found in muscles III, VI and VII, where the full motor units of two labelled axons were analysed, and in muscles II, IV and V in which the thickness of the muscle restricted our analysis to superficial regions where both labelled axons were optically accessible (Fig. 2). It was unlikely that the expression of fluorescent proteins in itself had any effect on these results because in two cases (muscles I and V) the CFP axon dominated and in two cases (muscles II and III) the YFP axon dominated (compare Figs 3a–h and 4a; see also ref. 19).

The similarity of innervation patterns at the cohort of neuromuscular junctions co-innervated by the same pair of motor neurons also extended to situations with no clear skewing. In these cases (motor units 5 versus 6, 8 versus 9, 10 versus 11, and 15 versus 16, Fig. 2; see also Supplementary Fig. 2a–c) two labelled inputs segregated to opposite sides of each co-innervated junction¹³ and on average neither had a significantly larger area (Fig. 2). The respective areas of synaptic contact at the co-innervated junctions were, however, significantly skewed relative to the rest of their motor units (column b, Fig. 2).

Two examples (muscle II and III) provided evidence that the final outcome of the competition was also strongly biased by the identity of the competitors. In these cases the weaker axon had either already lifted off the neuromuscular junction (Fig. 4a) or was a detached retraction bulb that had pulled back from the junction (see Supplementary Fig. 2f, g), even though on many other muscle fibres this same axon was the dominant branch when confronting different competitors (column b, Fig. 2). Thus the outcome of synaptic competitions for the branches of a particular axon was regulated by the company they keep, that is, the identity of the neuronal competitors.

One case appeared to provide a glimpse of a competitive hierarchy among axons. In muscle III, there were three distinctly labelled motor axons ('A', 'B' and 'C') that produced three stereotyped pair-wise sets of connections (summarized in Fig. 4b and Supplementary Fig. 2) in which axon A was dominant over B, B was equally matched by C, and A was dominant over C.

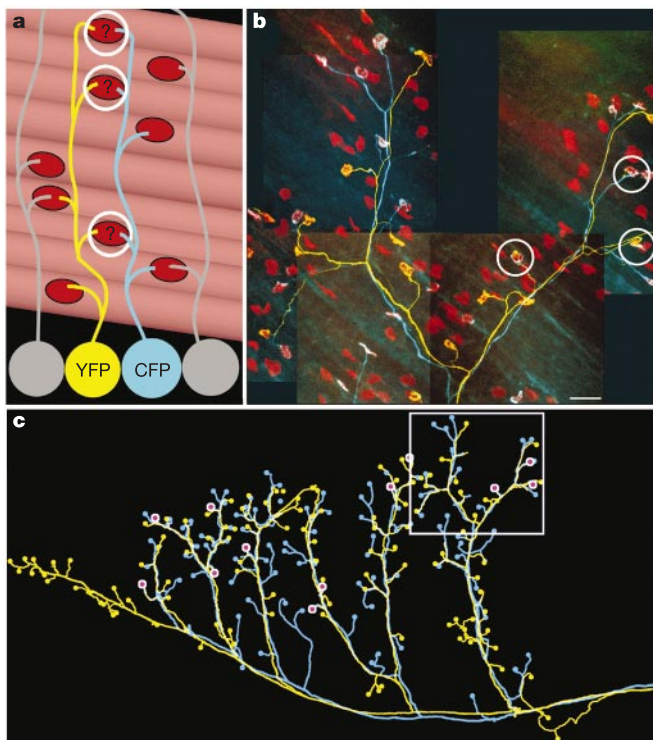


Figure 1 Reconstructions of two competing motor units in a developing muscle. **a**, Mice were bred to generate both a single CFP- and a YFP-expressing motor axon in the same muscle. The aim was to evaluate their relative contribution to co-innervated neuromuscular junctions (circles). **b**, A montage of confocal image stacks showing the CFP (blue) and YFP (yellow) axons in one region of the flexor digitorum profundus muscle (P8). Post-synaptic receptor sites are labelled with Alexa-594 conjugated bungarotoxin (BTX-red). Co-innervated junctions are circled. **c**, Drawing of the entire CFP (blue) and YFP (yellow) motor units. The neuromuscular junctions innervated by the YFP motor unit (yellow dots), the CFP motor unit (blue dots) or both ($n = 12$, purple dots) are widely distributed throughout the muscle. The boxed region is shown at high resolution in **b**. Scale bar, $50 \mu\text{m}$.

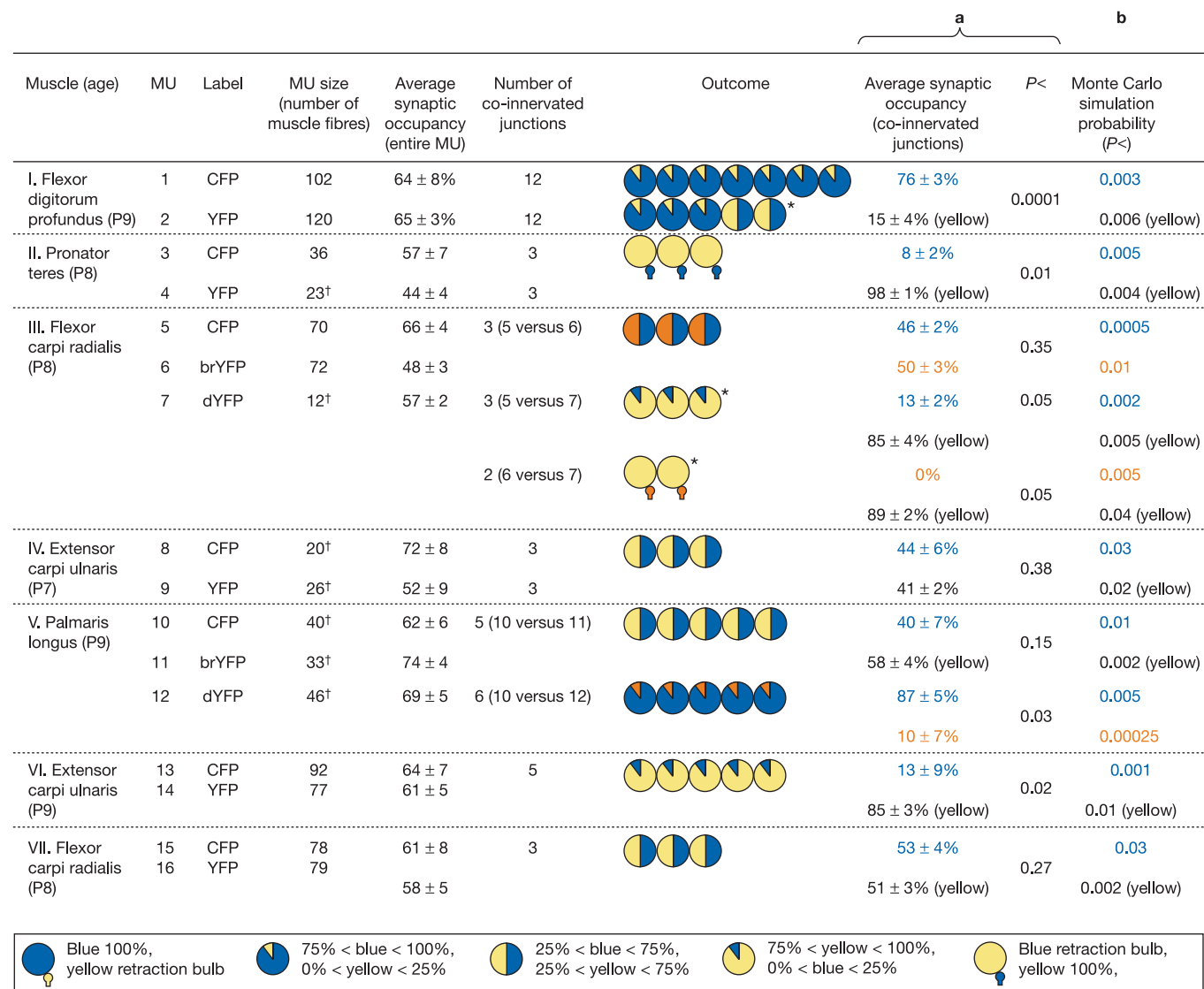


Figure 2 Double-coloured motor units in neonatal mice. MU, motor units; *, triply innervated junction; †, partial motor unit reconstructed.

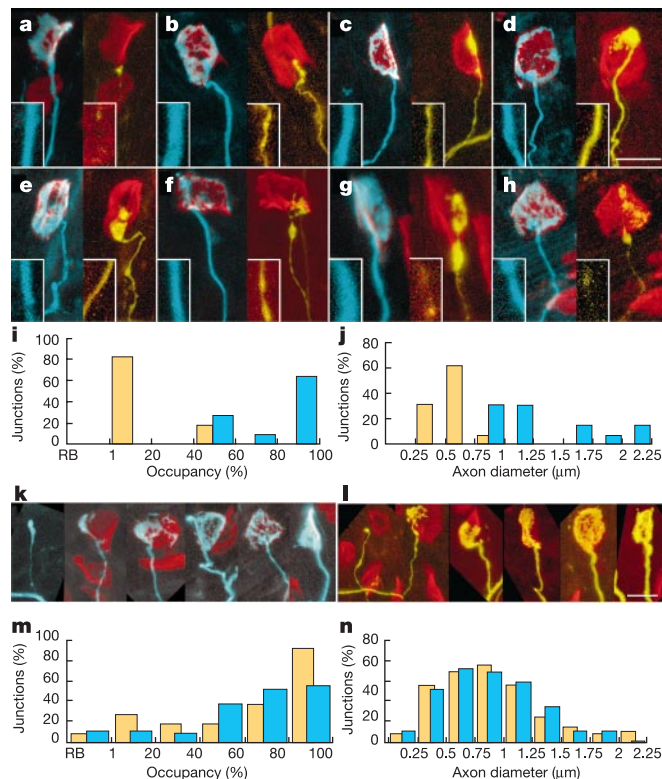
Interestingly, the pecking order of competitors corresponded to the relative sizes of their motor axon arborizations. In four cases (muscles I, III, VI and VII) with complete motor unit reconstructions, there was an inverse relation between competitive vigour at co-innervated neuromuscular junctions and the motor unit size of the two competing axons (Fig. 4c). In these cases, the axon with the larger motor unit occupied less synaptic territory at co-innervated postsynaptic targets than the axon with the smaller motor unit. Additionally, in cases where the two motor units were equivalent in size, their branches to co-innervated junctions were equivalent in terms of occupancy. These results argue that the relative amount of some resource skews the competitive vigour in favour of the smaller axonal arbor.

In conclusion, our results show that both the pace and outcome of synapse elimination are determined by the identity of the particular inputs co-innervating a target cell. Previous work has shown that during the process of synapse elimination the various branches of a motor axon undergo retraction from muscle fibres in an asynchronous fashion³. The studies presented here show, however, that each motor axon's branches can be subdivided into several different 'cohorts'. These cohorts are distinguished by the particular identity of the competing axon at co-innervated junctions and by

the fact that axonal branch appearance and fate within each cohort appears nearly identical. The similarity of axon branch behaviour when the competing axon is the same strongly suggests that stochastic or local factors play hardly any role in determining which axon branches ultimately survive. Moreover, the fact that the branches that confront a particular competitor appear so similar argues that the temporal aspects of synapse rearrangement are highly stereotyped. The asynchrony of branch retraction in a single motor unit³ thus appears to be the composite result of a series of highly synchronous synaptic reorganizations, each resolving at a different rate.

The similar appearance of the cohort of axonal branches that co-innervate postsynaptic cells shared by one other particular axon offers a unique view of many neuromuscular junctions at nearly the same stage of competition. In this way stages not previously recognized are now evident (see Supplementary Fig. 2).

These results may also provide insight into the factors that drive synaptic competition. Numerous experiments have shown that changes in synaptic activity can affect synapse competition¹⁴, and the relative activity of different synapses may be the crucial property that drives competition^{4,15}. By the second postnatal week, when pairs of axons typically innervate the same postsynaptic cell, it is



likely that the patterns or amounts of activity of the co-innervating motor axons are different^{16,17}. If asynchronous synaptic activation of the postsynaptic cell is the principal mechanism driving competition forward, then the result should be identical on all post-synaptic cells co-innervated by the same axons.

This hypothesis implies the existence of one axon with the ‘best’ activity pattern that consequently maintains all of its branches and another axon with the ‘worst’ activity pattern that withdraws from the muscle completely, neither of which has been observed^{8,9}. Alternatively, the relative competitive vigour of a neuron might depend both on the pattern of activity of its axon branches (which is fixed) and the efficacy of its synapses (which is malleable)¹⁸. Synaptic efficacy might, for example, be inversely related to the size of an axonal arbor. As an individual neuron withdraws at several post-synaptic target cells synchronously, it could subsequently redistribute synaptic resources to its remaining branches, increasing their competitive vigour and perhaps synchronously flipping the outcome in favour of inputs that were temporarily losing¹⁹. Conversely, axons that win numerous competitions early would

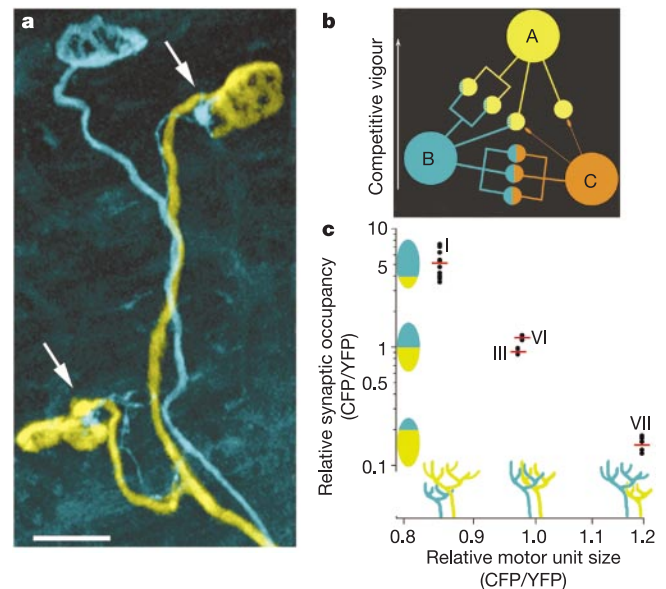


Figure 4 The identity of neuronal competitors determines the outcome of synapse elimination. **a**, Two neuromuscular junctions (arrows) contacted by the same YFP (yellow) axon have recently lost input from the same CFP axon (muscle II, Fig. 2). In both cases (and a third co-innervated junction, not shown) the CFP axon, in the final stages of synapse elimination, has lifted off the junction surface. Scale bar, 20 μm . **b**, Schematic of the connectivity of three motor units (A, B, C) showing the dominance of axon A over axon B, the similarity of axons B and C, and the dominance of A over C. These sets of interactions suggest a hierarchy in competitive vigour between the three axons (see Supplementary Fig. 2). **c**, Inverse relation between the skewing at individual co-innervated junctions and the relative sizes of the motor units of the two axons. Filled circles are individual neuromuscular junctions co-innervated by the two motor units (red lines, mean values; roman numerals refer to Fig. 2). Scale bar, 10 μm .

commit resources to these fully occupied junctions, as a result weakening branches engaged in unresolved competitions. Finally, inputs initially highly divergent in competitive vigour might become more evenly matched by such readjustments in the pecking order, making the outcome of competition at some junctions more protracted than at others.

In summary, the surprising power of the identity of the neuronal competitors to determine the outcome and pace of synaptic competitions argues that some unique attribute of each neuron can affect the behaviour of its widely distributed branches. A reasonable candidate for this attribute is the potency of synaptic excitation by the individual branches, which in turn is determined by the number of action potentials in the parent axon and the amount of transmitter released from the terminal. In support of this notion, the accompanying paper⁴ shows that decreasing synaptic efficacy strongly biases the outcome of competition at developing synapses. □

Methods

Mice

Transgenic mice (*thy1-YFP-H* and *thy1-CFP-S¹¹*) were bred together to produce double-subset-expressing mice. Mice were mated to produce timed pregnancies, and the day of birth was considered postnatal day 0 (P0).

Tissue preparation

Neonatal mice were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Forelimb muscles from both arms were dissected and post-fixed in 2% paraformaldehyde for 2 h. Muscles were rinsed in PBS, the connective tissue removed and the muscles were then incubated for 45 min in 5 $\mu\text{g} \mu\text{L}^{-1}$ Alexa-594-conjugated- α -bungarotoxin (Molecular Probes) diluted in 1% bovine serum albumin (BSA) in sterile lactated Ringer's solution. After rinsing, muscles were mounted on slides in Vectashield (Vector Laboratories).

Imaging and analysis

Motor units and neuromuscular junctions were reconstructed on laser scanning confocal microscopes (Olympus Flouview FV500 and Bio-Rad 1024). Images were obtained with a $\times 60$ (1.4 NA) oil objective with a zoom equal to 2 for motor unit reconstructions and zoom equal to 4 for diffraction-limited imaging of individual junctions. YFP was excited with the 488 nm line of an argon laser and detected with a 520–550 nm band-pass emission filter. CFP was excited with the 442 nm line of an HeCd laser and detected with a 465–495 nm band-pass emission filter. Alexa-594 α -bungarotoxin was excited by using the 568 line of a Krypton laser and detected with a 585 long-pass barrier filter. Z-steps were 0.6 μm for motor unit reconstructions and 0.3 μm for high-resolution imaging of individual junctions.

Z-stacks were flattened by using a two-dimensional maximum projection algorithm in MetaMorph (Universal Imaging Corporation), and montages of the 20–50 stacks that constitute a motor unit were aligned with Adobe Photoshop. Percentage occupancy at individual junctions was determined in MetaMorph by counting the number of YFP (or CFP) pixels overlying receptors labelled with Alexa-594-conjugated α -bungarotoxin. Axon diameter was determined, by using MetaMorph, as the width of the innervating CFP or YFP axon 10 μm from the edge of the endplate where the axon enters. Branch number was determined by constructing complete branch diagrams of the motor units of interest and counting the number of branch points between the cell body and a particular junction.

Pair-wise statistical comparisons between motor units (i.e. CFP occupancy versus YFP occupancy) were done by using a non-parametric Mann–Whitney non-directional test. Distributions of histograms (Fig. 3 and Supplementary Fig. 1) were compared by using a Kolmogorov–Smirnov (K–S) goodness-of-fit test with S-Plus 4, MathSoft. We used a Monte Carlo simulation (column b, Fig. 2) to test whether the distribution of synaptic occupancy of a specific cohort of junctions facing the same competitor was different than the overall distribution of synaptic occupancy of the motor unit containing that cohort. As the most striking characteristic of the co-innervated junctions was their apparent similarity to each other, we compared the standard deviation of synaptic occupancy of junctions or axon diameter within the cohort with the standard deviation of the entire motor unit. Specifically, one trial of the Monte Carlo simulation randomly selected the same number of junctions from the entire motor unit as observed in the co-innervated cohort, and the standard deviation of that random sample was calculated (program written by S. Turney for Matlab). The process was repeated 100,000 times. The probability that the homogeneity occurring within a cohort could have occurred by chance was taken as the percentage of randomly selected samples that had an equal or smaller standard deviation than the cohort.

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Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition

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Synaptic activity drives synaptic rearrangement in the vertebrate nervous system; indeed, this appears to be a main way in which experience shapes neural connectivity^{1,2}. One rearrangement that occurs in many parts of the nervous system during early post-natal life is a competitive process called 'synapse elimination'^{3,4}. At the neuromuscular junction, where synapse elimination has been analysed in detail, muscle fibres are initially innervated by multiple axons, then all but one are withdrawn and the 'winner' enlarges^{4–6}. In support of the idea that synapse elimination is activity dependent, it is slowed or speeded when total neuromuscular activity is decreased or increased, respectively^{4,7–13}. However, most hypotheses about synaptic rearrangement postulate that change depends less on total activity than on the relative activity of the competitors^{1–4,13,14}. Intuitively, it seems that the input best able to excite its postsynaptic target would be most likely to win the competition, but some theories and results make other predictions^{14–18}. Here we use a genetic method to selectively inhibit neurotransmission from one of two inputs to a single target cell. We show that more powerful inputs are strongly favoured competitors during synapse elimination.

The only two experiments that have differentially decreased activity of some inputs to a muscle led to opposite conclusions: the more active axon was favoured in one, and the less active in the other^{17,18}. To re-examine this issue, we reduced neurotransmission from a subset of motor axons by depleting them of choline acetyltransferase (ChAT), the sole synthetic enzyme for the neurotransmitter, acetylcholine. Neuromuscular junctions (NMJs) form in mutant mice that completely lack ChAT (*ChAT*^{−/−}), but electrophysiological recordings showed that no neurotransmission occurs, and the mice die at birth^{19,20}. Here, we used a conditional allele of the *ChAT* gene (*ChAT*^{lox/flox}), so that we could allow embryogenesis to proceed normally, then inactivate *ChAT* postnatally. Exons near the 5' end of ChAT were flanked by loxP sites; excision by Cre recombinase generated the *ChAT*^{−/−} allele¹⁹. To

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control the timing and magnitude of excision, Cre was fused to the ligand-binding domain of an oestrogen receptor (ER); the ER domain prevents Cre activity, but inhibition is relieved by administration of ligand²¹. To further control Cre activity, the receptor fragment had been mutated to prevent binding of the endogenous ligand (oestradiol) while retaining sensitivity to a synthetic ligand (tamoxifen). In the transgenic mice we used (*CX-CreER*; ref. 22), the Cre-ER fusion was expressed nearly ubiquitously. Thus, we anticipated that administration of tamoxifen to *CX-CreERxChAT^{flox/flox}* or *CX-CreERxChAT^{flox/-}* pups would inactivate ChAT in subsets of motor neurons, with the number of affected cells being dependent on the dose of tamoxifen administered.

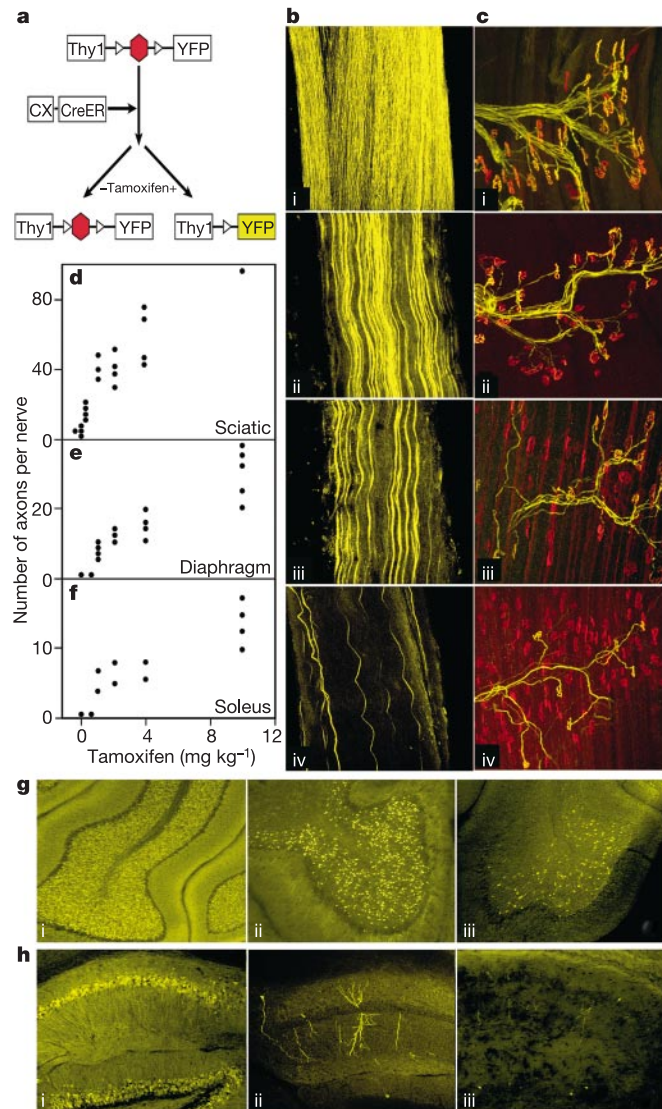


Figure 1 Activation of gene expression in subsets of motor neurons. **a**, Neuron-specific regulatory elements from the *thy1* gene drive expression of YFP after Cre-mediated excision of a STOP cassette. Cre was fused to the ligand-binding domain of a steroid receptor (CreER), so excision required administration of tamoxifen. **b, c**, Sciatic nerves (**b**) and sternomastoid muscles (**c**) from *Thy1-STOP-YFPx-CreER* mice (**i**) or *Thy1-STOP-YFPx-CX-CreER* mice (**ii–iv**). Tamoxifen was injected at 10 mg kg⁻¹ (**ii**), 4 mg kg⁻¹ (**iii**) or 0.5 mg kg⁻¹ (**iv**), 10–15 d before analysis. Muscles in (**c**) were stained with anti-GFP plus rhodamine α -bungarotoxin to label the acetylcholine receptors at endplates. **d–f**, YFP-positive axons as a function of tamoxifen dose in *Thy1-STOP-YFPx-CX-CreER* mice. Each point represents one muscle or nerve. **g, h**, Cerebella (**g**) and hippocampi (**h**) from *Thy1-STOP-YFPx-CX-CreER* mice injected with 10 mg kg⁻¹ (**i**), 1 mg kg⁻¹ (**ii**) or 0 mg kg⁻¹ (**iii**) tamoxifen. At the lowest doses of tamoxifen, Golgi-like labelling of individual hippocampal neurons is evident. Fewer than 1% of granule cells are labelled without tamoxifen.

To test this strategy, we first generated transgenic mice in which expression of a yellow fluorescent protein (YFP) in neurons²³ was completely dependent on Cre-mediated excision of inhibitory (STOP) sequences (Fig. 1a). We mated these *Thy1-STOP-YFP* mice to mice in which constitutively active Cre was expressed throughout the embryo (*A-Cre*; ref. 24) and selected lines in which all motor axons of *Thy1-STOP-YFPx-A-Cre* offspring were YFP-positive (Fig. 1b(i)). These lines were then used to test the ability of the *CX-CreER* transgene to act in motor neurons. The number of labelled axons in peripheral nerves of *Thy1-STOP-YFPx-CX-CreER* mice was proportional to the dose of tamoxifen administered, but the intensity with which individual axons were labelled did not appear to vary with dose (Fig. 1b–f). Expression of YFP in many populations of central neurons was also tamoxifen dependent (Fig. 1g), generating a ‘Golgi-like’ pattern that may be useful for imaging studies. Based on these results, we chose doses of 5 and 100 μ g (approximately 2.5 and 50 mg kg⁻¹, referred to below as ‘low’ and ‘high’) administered on the first postnatal day (P0), to excise floxed sequences in 10–20% or >50% of motor neurons, respectively.

Next, we assessed the ability of the *CX-CreER* transgene to inactivate ChAT. Motor axons were labelled by antibodies to ChAT in *CreERxChAT^{flox/flox}* mice without tamoxifen, but labelling was lost after administration of tamoxifen at P0. As expected, 10–20% and >50% of axons in intramuscular nerves of *CX-CreERxChAT^{flox/flox}* mice were ChAT-negative (ChAT⁻) 1 week after low and high doses of tamoxifen, respectively (Fig. 2a, b). Similar results were observed in soleus, diaphragm, sternomastoid, serratus anterior, spinotrapezius and extensor digitorum longus muscles. No behavioural abnormalities were detected after administration of the low dose, but mice became noticeably weak by P9 and died at P14–15 after administration of the high dose. Importantly, a high dose of tamoxifen neither led to weakness nor affected neuromuscular morphology in *CX-CreERxChAT^{flox/+}* or *CX-CreERxThy1-STOP-YFP* mice (data not shown).

Based on these results, we assessed NMJs at P11 in *CX-CreERxChAT^{flox/flox}* mice that had received a low dose of tamoxifen at P0. In the muscles examined, all muscle fibres are multiply innervated at P0, then lose inputs during the first two postnatal weeks^{4,5}. Most NMJs have already completed synapse elimination by P11, but we chose this age for two reasons: (1) by immunohistochemical criteria, most axons were unambiguously either ChAT⁺ or ChAT⁻ by this time; (2) at this age, the ‘winning’ and ‘losing’ inputs can be predicted with ~80% accuracy by assessing which occupies more territory (ref. 6 and M. K. Walsh, personal communication). Whole muscles were triply labelled with fluorescent- α -bungarotoxin (which binds to acetylcholine receptors in the postsynaptic membrane), antibodies to ChAT, and a mixture of antibodies to neurofilaments (to label axons) and a synaptic vesicle protein (to label motor nerve terminals). NMJs were imaged in whole mounts and reconstructed by confocal microscopy.

The main result is illustrated in Fig. 2c, which shows a group of three neighbouring NMJs from a low dose *CX-CreERxChAT^{flox/flox}* mouse. This group was unusual in that all three NMJs were doubly innervated, even though ~80% of the fibres in this muscle were already singly innervated. At two of the NMJs, one axon was ChAT⁺ and the other was ChAT⁻. Knowing that the two axons occupy largely non-overlapping territories at this stage (see Table 1 in ref. 25), we compared the ChAT-stained area of the terminal arbor (representing the ChAT⁺ territory) with the neurofilament and vesicle-positive area (representing both axons). At both of these synapses, the ChAT⁺ axon occupied more than half of the area occupied by both axons together: 75% in the NMJ shown at higher power in Fig. 2d, and 81% in its neighbour. The average occupancy by ChAT⁺ axons at such doubly innervated sites was $84.9 \pm 2.2\%$ (mean $\pm$ s.e.m., $n = 29$ synapses from four muscles). Similar results ($82 \pm 2.1\%$ occupancy) were obtained when the area of the ChAT⁺ axons was expressed as a fraction of the acetylcholine

receptor-rich area rather than as a fraction of the total presynaptic arbor. By either measure, the ChAT⁺ axon occupied more than 50% of the site at all 29 synapses (Fig. 2g; non-random at $P \ll 0.001$ by χ^2 test). Similar results were obtained after tamoxifen injection at P2 (data not shown).

These results imply that relative efficacy biases the outcome of synaptic competition. Before drawing this conclusion, however, we considered two alternative explanations. First, ChAT⁺ axons were in the minority, and it was possible that successful axons were those whose activity level conformed most closely to the average level in the target field (a 'democratic' model). Second, inactive axons might be intrinsically incompetent to maintain a synapse, regardless of whether or not they were pitted against a more active axon. To address these possibilities, we confocally reconstructed synapses from high-dose CX-CreERxChAT^{fllox/fllox} mice, in which most axons were ChAT⁺ (Fig. 2e). At all synapses innervated by a ChAT⁺ and a

ChAT⁺ axon, the ChAT⁺ axon occupied more than 50% of the site (average $79.9 \pm 5.8\%$; Fig. 2h), indicating that activity level predicted outcome whereas majority status did not. Moreover, multiple innervation was more prevalent in these muscles than in controls (Fig. 2i), and numerous sites were innervated by two ChAT⁺ axons, consistent with previous results from globally paralysed muscles^{6,8,11}, but inconsistent with the idea that all ChAT⁺ axons withdraw from synapses. ChAT⁺ axons also fully occupied synaptic sites at singly innervated NMJs; in these cases, competition may have been underway or complete before ChAT was lost. Thus, inactive axons are capable of maintaining synaptic territory, but appear to be hampered from doing so in the presence of an active competitor.

A second aspect of the competitive process allowed us to further test the idea that ChAT⁺ axons fare poorly not because they are inactive, but rather because they are pitted against more efficacious

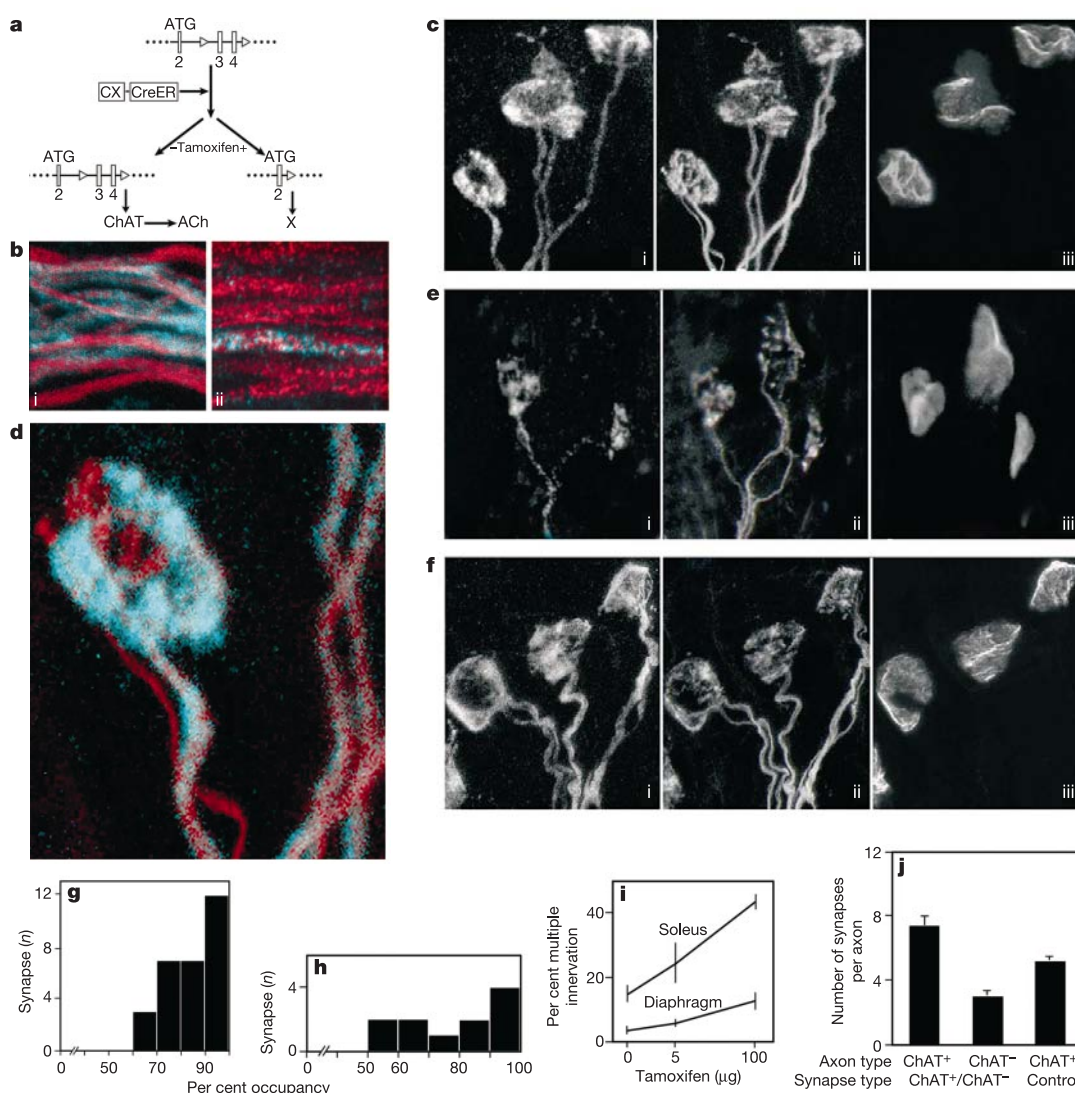


Figure 2 ChAT⁺ axons are favoured competitors over ChAT⁺ axons at multiply innervated neuromuscular junctions. **a**, The conditional *Chat* allele (*Chat*^{fllox/fllox}), and the null allele¹⁹ generated by Cre-mediated excision after tamoxifen administration to CX-CreERxChAT^{fllox} mice. **b**, Nerve branches from CX-CreERxChAT^{fllox/fllox} mice treated with 5 µg (**i**) or 100 µg (**ii**) tamoxifen at P0, then stained for neurofilaments (red) and ChAT (blue) at P11. **c–f**, neuromuscular junctions (NMJs) from CX-CreERxChAT^{fllox/fllox} mice administered 5 µg (**c**, **d**) or 100 µg (**e**, **f**) tamoxifen or saline (**f**) at P0, then triply stained for axons (**i**), ChAT (**ii**) and acetylcholine receptors (**iii**) at P11. ChAT (blue) and axon (red) staining from one NMJ are overlaid in **d**, **g**, **h**. Fraction of the synaptic site occupied by the

ChAT⁺ axon at NMJs co-occupied by a ChAT⁺ and a ChAT⁺ axon after administration of 5 µg (**g**) or 100 µg (**h**) tamoxifen. The ChAT-positive axon occupied more than 50% of all sites. **i**, Percentage of NMJs that were doubly innervated at P11 in soleus or diaphragm muscles from CX-CreERxChAT^{fllox/fllox} mice administered 0, 5 or 100 µg tamoxifen at P0. Bars indicate range; ≥ 100 NMJs were counted from each muscle. **j**, Synaptic territory of ChAT⁺ or ChAT⁺ axons, calculated as the ratio of the number of NMJs innervated by each category of axon to the number of axons of that category in an intramuscular nerve branch. (Mean $\pm$ s.e.m. for 10–18 microscope (20 $\times$) fields.)

opponents. During naturally occurring synapse elimination, the axon branch leading to the 'losing' terminal atrophies as or before the terminal branches withdraw^{25,26}. We therefore measured the calibre of pre-terminal axons in *CX-CreERxChAT^{fllox/fllox}* mice. At synapses innervated by a ChAT⁺ and a ChAT⁻ axon, the ChAT⁺ axon was usually thicker and never thinner than the ChAT⁻ axon, providing an additional line of support for the conclusion that impotent axons fare poorly in the competition (Fig. 3a–c). More importantly, the difference between the diameters of the two inputs was greater at NMJs innervated by a ChAT⁺ and a ChAT⁻ axon (where activity differences between inputs were maximized) than at NMJs innervated by two ChAT⁻ axons (where activity differences between inputs should be less) (Fig. 3d). This result does not reflect

a general atrophy of ChAT⁻ axons, because the average diameter of axons at synapses with two ChAT⁻ inputs was similar to that at synapses with two ChAT⁺ inputs (Fig. 3e). Moreover, axons at synapses innervated by two ChAT⁺ or two ChAT⁻ inputs were on average thinner than the ChAT⁺ axons and thicker than the ChAT⁻ axons at synapses with one input of each type. In other words, a ChAT⁻ axon fared worse when confronting a normally active axon than when confronting another weak input. This result shows that the size of an axon branch is influenced by the identity of the competing input, implying that strong inputs destabilize weaker inputs to a common postsynaptic target.

Finally, we tested the prediction that, if active inputs out-compete less active inputs, active motor axons should come to innervate more muscle fibres than inactive motor axons. We had no way of determining the absolute size of motor units, so we counted, in multiple microscope fields, the numbers of ChAT⁺ and ChAT⁻ axons in intramuscular nerves and the numbers of NMJs with ChAT⁺ or ChAT⁻ terminals. By this measure, each ChAT⁺ axon innervates over twice as many synaptic sites as each ChAT⁻ axon (Fig. 2i; only one NMJ in this data set was innervated by both a ChAT⁺ and a ChAT⁻ axon). The number of NMJs per axon in control muscles, measured in the same way, was intermediate between these values. This result confirms the inference that differences in efficacy bias not only the progress of competition at individual sites, but also the outcome of that competition.

In summary, we have directly tested the influential hypothesis^{1–18} that differential efficacy affects the competitive processes that result in the elimination of supernumerary synapses during early post-natal life. The test relied on three techniques. First, targeting of ChAT allowed us to decrease neurotransmission specifically, without affecting other aspects of axonal physiology such as propagation of action potentials. Second, the tamoxifen-Cre system allowed us to inactivate some inputs to a muscle, or even to a single postsynaptic cell, without perturbing neighbouring inputs. Importantly, in the 'low dose' animals, ChAT⁻ axons were in a small minority, so potentially complicating global effects on activity were inconsequential. Third, confocal imaging allowed us to measure synaptic occupancy on individual postsynaptic cells, rather than inferring occupancy from electrical or mechanical measurements as had been done previously^{17,18}. By combining these methods, we showed for the first time that the more powerful input to a synaptic target cell destabilizes a less powerful input to the same target. It remains to be determined whether the timing and pattern of neurotransmission, as well as its total amount, influence the outcome of this competitive process^{1,2,13,14,27,28}.

Our results leave open the question of whether activity is the predominant driver of synapse elimination under normal circumstances, or whether it plays more subtle roles. If activity predominates, one might imagine that all synapses at which branches of the same two axons compete would proceed along the same path to the same outcome. Moreover if motor units in normal muscles have limited resources, larger motor units might release less transmitter at each synapse and be at a competitive disadvantage. A companion study²⁵ confirms both predictions. Taken together, those results and ours lead to the surprising idea that inter-axonal differences in the ability to an axon to activate a target (reflecting levels or patterns of axonal activity and the amount of transmitter each impulse releases) can completely account for the tempo and outcome of some synaptic rearrangements. □

Methods

Mice

Transgenic mice expressing YFP under the control of neuron-specific regulatory elements from the murine *thy-1* gene are described in ref. 23. To generate transgenic mice that expressed YFP conditionally, we modified the vector by inserting a 'lox-stop-lox' cassette²⁹ upstream of the first codon of YFP. Transgenic mice expressing Cre under the control of regulatory sequences from the β -actin gene (*A-Cre*) were a gift from G. Martin (University of

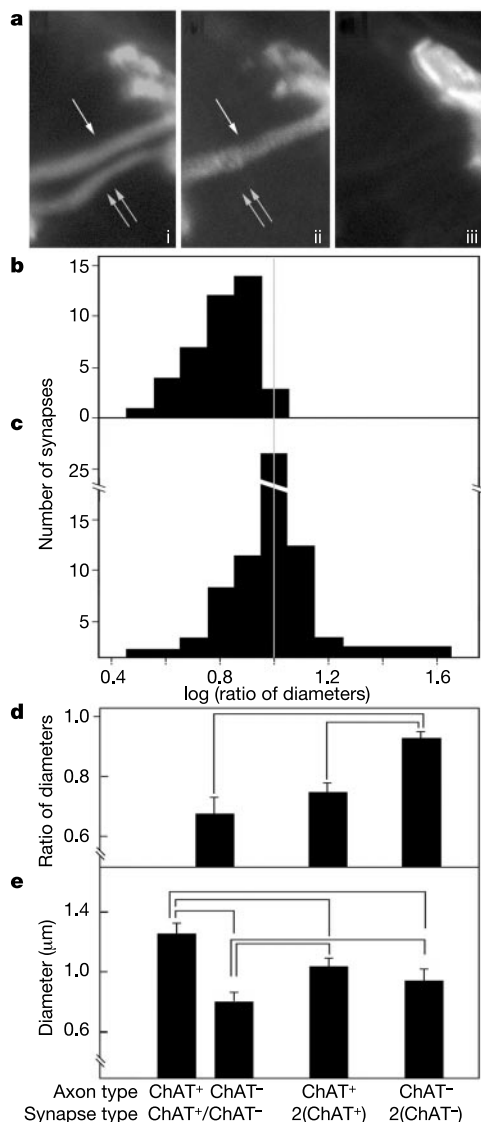


Figure 3 ChAT⁻ axons fare worse when pitted against a ChAT⁺ axon than when pitted against another ChAT⁻ axon. **a**, Pre-terminal axons at an NMJ innervated by a ChAT⁺ and a ChAT⁻ axon, triply labelled as in Fig. 2c–f. The ChAT⁺ axon is thicker than the ChAT⁻ axon. **b**, Ratio of the pre-terminal diameter of the ChAT⁻ axon to that of the ChAT⁺ axon at synaptic sites co-occupied by one axon of each type. **c**, Similar measurements of ratio of diameters of thin to thick axons at sites in the same muscles co-occupied by two ChAT⁺ or two ChAT⁻ axons. At each site, one diameter was assigned at random as the numerator. **d**, **e**, Pre-terminal diameters of axons co-occupying individual synaptic sites (**e**), and ratio of the diameter of the thinner axon at each site to that of the thicker (**d**). Values joined by horizontal lines differ from each other at $P < 0.05$ by Student's *t*-test. Analysis is based on 40 ChAT⁺/ChAT⁻, 45 ChAT⁺/2(ChAT⁺) and 18 ChAT⁻/2(ChAT⁻) synapses.

California at San Francisco)²⁴. Transgenic mice expressing Cre-ER nearly ubiquitously under the control of hybrid viral and actin regulatory elements (*CX-CreER*) are described in ref. 22.

To target the *ChAT* locus, exons 3 and 4 were flanked by loxP sites and a *Neo* gene was introduced in the intron between exons 4 and 5 (ref. 19). In ref. 19, we showed that excision of these exons in the germline by mating to *A-Cre* mice generated a null allele in subsequent generations, in which no ChAT protein or activity was detectable. Because we were concerned that the presence of foreign sequences in the ChAT locus might interfere with ChAT expression, the *Neo* gene was flanked by Frt sites, so that it could be excised by mating to mice in which Flp recombinase was expressed ubiquitously³⁰. However, we detected no difference in phenotype between mice that retained or lacked the *Neo* gene. All mice were maintained in a C57/B6 background. Tamoxifen (Sigma) was dissolved in 0.1% ethanol, 99% corn oil for intraperitoneal injection.

Imaging

Mice were perfused with 2% paraformaldehyde, then muscles were dissected, postfixed for 30 min in the same solution, and treated successively with 0.1M glycine, 0.5% Triton-X-100 (2 h) and 4% bovine serum albumin, 0.5% Triton-X-100 in PBS (2 h; blocking solution). They were then stained for 24 h with primary antibodies (mouse anti-neurofilament (Sternberger), mouse anti-SV2 (Developmental Studies Hybridoma Bank) and goat anti-ChAT (Chemicon)), washed for 24–48 h, stained for 24 h with fluorescent reagents (Alexa 594–donkey anti mouse IgG, Alexa 488–donkey anti-goat IgG, and Alexa 647- α -bungarotoxin, all from Molecular Probes), washed for 24 h, and mounted. Stacks of images were collected at intervals of 0.35–0.5 μ m with a confocal microscope, then reconstructed in Metamorph (Universal Imaging) and rendered in Photoshop (Adobe).

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Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels

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TRPV4 is a widely expressed cation channel of the 'transient receptor potential' (TRP) family¹ that is related to the vanilloid receptor VR1 (TRPV1). It functions as a Ca²⁺ entry channel² and displays remarkable gating promiscuity by responding to both physical stimuli (cell swelling, innocuous heat^{2–7}) and the synthetic ligand 4 α PDD⁸. An endogenous ligand for this channel has not yet been identified. Here we show that the endocannabinoid anandamide and its metabolite arachidonic acid activate TRPV4 in an indirect way involving the cytochrome P450 epoxygenase-dependent formation of epoxyeicosatrienoic acids. Application of 5',6'-epoxyeicosatrienoic acid at submicromolar concentrations activates TRPV4 in a membrane-delimited manner and causes Ca²⁺ influx through TRPV4-like channels in vascular endothelial cells. Activation of TRPV4 in vascular endothelial cells might therefore contribute to the relaxant effects of endocannabinoids and their P450 epoxygenase-dependent metabolites on vascular tone^{9–12}.

To search for potential endogenous activators of TRPV4, we monitored cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) signals in HEK-293 cells expressing TRPV4 in response to various substances related to 4 α -phorbol-12,13-didecanoate (4 α PDD), a potent synthetic ligand of TRPV4, and to anandamide (arachidonylethanolamide (AEA)), a lipid activator of TRPV1. Arachidonic acid (AA; 10 μ M), a metabolite of AEA, caused a robust increase in [Ca²⁺]_i in transfected cells but had only a marginal effect in non-transfected cells (Fig. 1a). The elevation of [Ca²⁺]_i in TRPV4-transfected cells was inhibited by the TRPV4 blocker ruthenium red (10 μ M)⁸ (Fig. 1b) and depended on extracellular Ca²⁺, indicating that AA activates Ca²⁺ entry through TRPV4 channels.

In whole-cell patch-clamp experiments, AA activated a large, outwardly rectifying current in TRPV4-transfected cells but not in non-transfected cells (Fig. 1c, d). This current reversed at positive potentials (+21 $\pm$ 3 mV, n = 42, 30 mM external Ca²⁺ concentration ([Ca²⁺]_e))^{7,8}, and was blocked in a highly voltage-dependent manner by ruthenium red (RR). These properties are indistinguishable from those of the current activated by the synthetic TRPV4

agonist 4 α PDD^{8,13}. The AA concentration for half-maximal activation was $2.0 \pm 0.4 \mu\text{M}$. AA also activated single-channel currents in cell-attached patches from TRPV4-transfected cells, but not from non-transfected cells (Fig. 1e, f). The amplitude of the single-channel current at -60 mV was $-3.57 \pm 0.17 \text{ pA}$ (chord conductance $59.5 \pm 2.8 \text{ pS}$, from six cells), which is identical to that of the 4 α PDD-activated TRPV4 channel⁷.

Hydrolysis of the endocannabinoids AEA and 2-arachidonoylglycerol (2-AG), which are endogenous ligands of the CB1 and CB2 metabotropic cannabinoid receptors, results in the formation of AA. AEA is exclusively hydrolysed by the enzyme fatty acid amide hydrolase (FAAH), whereas hydrolysis of 2-AG can also occur through monoacylglycerol lipase and other esterases¹⁴. Like AA, AEA and 2-AG increased $[\text{Ca}^{2+}]_i$ in TRPV4-expressing cells, but not in non-transfected cells (Fig. 2a). Both compounds also activated TRPV4 currents in about one-quarter of TRPV4-expressing cells (7 of 32 cells; Fig. 2b–h), which never occurred in non-transfected cells (46 cells; Fig. 2h).

Next we investigated whether the activation of TRPV4 by AEA requires its metabolic conversion to AA. Methanandamide ($10 \mu\text{M}$), a non-hydrolysable derivative of AEA, failed to increase $[\text{Ca}^{2+}]_i$ or to activate currents in TRPV4-transfected cells. It also inhibited the AEA-activated TRPV4 current by $91 \pm 1\%$ ($n = 11$), which is consistent with its action as a non-selective inhibitor of AEA-metabolizing enzymes. Preincubation of TRPV4-expressing cells with the selective FAAH inhibitor phenylmethylsulphonyl fluoride ($100 \mu\text{M}$) also abolished the AEA-induced increase in $[\text{Ca}^{2+}]_i$, but not that induced by AA. These results indicate that activation of

TRPV4 by AEA requires its FAAH-dependent hydrolysis to AA.

In contrast to 4 α PDD, which activates TRPV4 in a membrane-delimited manner⁷, AA, AEA and 2-AG failed to activate TRPV4 channels when applied to the cytosolic side of cell-free inside-out patches ($n \geq 10$). In addition, 5,8,11,13-eicosatetraynoic acid (ETYA), a non-metabolizable analogue of AA, neither increased $[\text{Ca}^{2+}]_i$ nor activated a current. We therefore looked for an activator among the metabolites of AA. Figure 3a schematically shows the signal transduction cascade upstream and downstream of AA. Because inhibition of FAAH prevented activation by AEA completely, we excluded lipoxygenase products of AEA as activators of TRPV4 and screened the lipoxygenase, cyclo-oxygenase and cytochrome P450 metabolites of AA as potential activators of TRPV4 (refs 15, 16).

ETYA ($10 \mu\text{M}$) abolished the Ca^{2+} increase and current activated by AA, which is consistent with its action as a non-specific blocker of all AA-metabolizing enzymes. The AA-induced increase in $[\text{Ca}^{2+}]_i$ was insensitive to indomethacin ($50 \mu\text{M}$), nordihydroguaiaretic acid (20 or $50 \mu\text{M}$) and the combination of both inhibitors (Fig. 3b), which rules out the cyclo-oxygenase and lipoxygenase

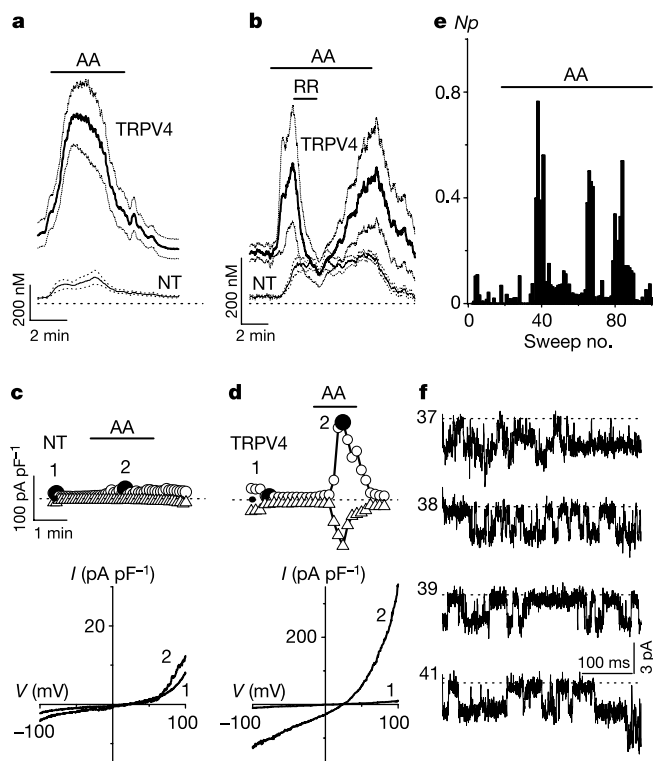


Figure 1 Effect of AA on $[\text{Ca}^{2+}]_i$ and currents in TRPV4-expressing HEK-293 cells. **a**, Time course of the changes in $[\text{Ca}^{2+}]_i$ induced by $10 \mu\text{M}$ AA (means $\pm$ s.e.m.) in TRPV4-transfected (thick lines, TRPV4) and non-transfected cells (thin lines, NT). **b**, Effect of ruthenium red (RR, $10 \mu\text{M}$) on the effect of AA. **c**, **d**, Upper panels, effect of $10 \mu\text{M}$ AA on the whole-cell current at -80 mV (triangles) and $+80 \text{ mV}$ (circles) in non-transfected (**c**) and in TRPV4-transfected (**d**) cells (holding potential 0 mV ; $[\text{Ca}^{2+}]_o = 30 \text{ mM}$). Lower panels, current–voltage relations at the corresponding times in the top panels. **e**, Single-channel activity induced by $10 \mu\text{M}$ AA (cell-attached, average current per sweep). **f**, Representative current traces corresponding to the sweep numbers in **e**.

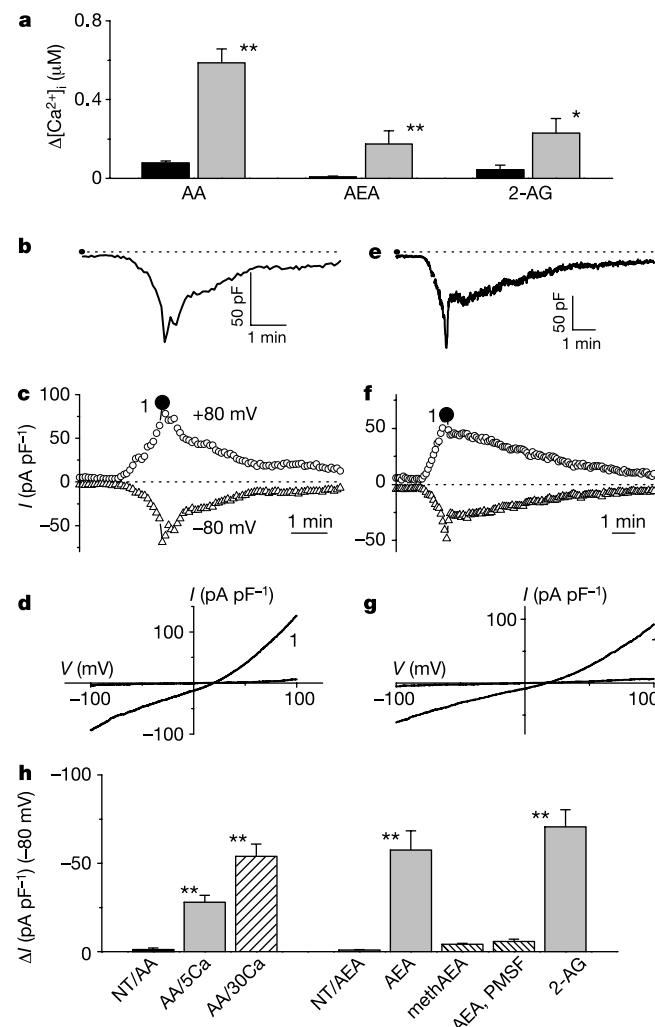


Figure 2 AEA and 2-AG increase $[\text{Ca}^{2+}]_i$ and activate currents in TRPV4-transfected cells. **a**, Increase by $10 \mu\text{M}$ AA, AEA and 2-AG of $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$) in non-transfected (black) and TRPV4-transfected (grey) cells. **b**, **e**, Inward current elicited by $10 \mu\text{M}$ AEA (**b**) and $10 \mu\text{M}$ 2-AG (**e**) in TRPV4-transfected but not in non-transfected cells (holding potential 0 mV ; $[\text{Ca}^{2+}]_o = 5 \text{ mM}$). **c**, **f**, Time course of the current at -80 and $+80 \text{ mV}$, under the same conditions as in **b** and **e**. **d**, **g**, I – V curves at the times indicated in **c** and **f** (filled circles). **h**, Current densities at -80 mV in the presence of various agonists ($10 \mu\text{M}$, data for AA obtained at 5 and 30 mM $[\text{Ca}^{2+}]_o$; all other data at 5 mM). NT, non-transfected; PMSF, phenylmethylsulphonyl fluoride.

pathways. In contrast, miconazole, an inhibitor of P450 epoxygenase, and 17-octadecynoic acid (17-ODYA), an inhibitor of the P450 epoxygenase and ω/ω -1-hydroxylases¹⁶, each completely abolished the AA-induced increase of $[Ca^{2+}]_i$ in TRPV4-transfected cells (Fig. 3b) at concentrations of 10 μ M. Concentrations for half-maximal inhibition of the AA-induced increase of $[Ca^{2+}]_i$ were 571 ± 207 nM and 352 ± 112 nM for miconazole and 17-ODYA, respectively. The cytochrome P450 inhibitors ETYA, miconazole and 17-ODYA did not significantly affect the increased basal $[Ca^{2+}]_i$ level in TRPV4-transfected cells (Fig. 3c), which represents, as reported previously, the basal TRPV4 channel activity in the absence of agonist^{7,8}. Similarly, responses to 4α PDD were preserved in the presence of these blockers (Fig. 3d), indicating that these drugs exert their inhibitory effect mainly through inhibition of cytochrome P450 rather than by a direct blocking effect on the TRPV4 channel.

Because these findings indicate that AA metabolites formed in the epoxygenase pathway might be involved in the activation of TRPV4, we tested whether epoxyeicosatrienoic acids (EETs) are able to

activate TRPV4. Bath application of 500 nM 5',6'-EET increased $[Ca^{2+}]_i$ (the concentration giving half-maximal response was 130 ± 86 nM) in TRPV4-transfected but not in non-transfected cells. This increase was also dependent on extracellular Ca^{2+} (Fig. 4d) (Fig. 4a–c). Significant but much smaller responses were observed with 8',9'-EET ($\Delta[Ca^{2+}]_i$ 178 ± 85 nM at 500 nM), whereas 11',12'-EET, 14',15'-EET and 20-hydroxyeicosatetraenoic acid were ineffective, suggesting that the cytochrome P450 ω/ω -1-hydroxylases are not involved in TRPV4 activation. Applying 5',6'-EET to inside-out patches of TRPV4-transfected cells induced single-channel activity with a chord conductance of 60.5 ± 2.5 pS ($n = 4$), similar to that of TRPV4 channels activated by 4α PDD or heat⁷ (Fig. 4e). Single-channel responses were never detected in non-transfected cells ($n = 5$). Bath application of 5 μ M 5',6'-EET also activated a whole-cell current with typical TRPV4 features (Fig. 4f–h). These data show that 5',6'-EET opens TRPV4 channels in a membrane-delimited fashion.

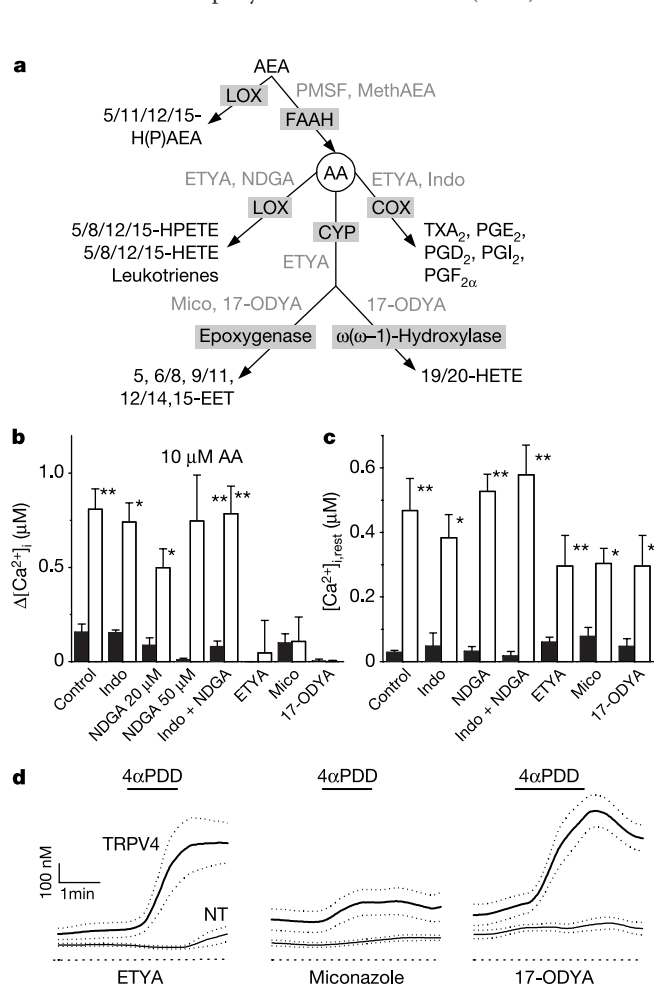


Figure 3 Unravelling the activation cascade of TRPV4. **a**, Signalling cascade for metabolism of AEA, including the metabolizing enzymes (grey boxes) and corresponding blockers. COX, cyclo-oxygenase; CYP, cytochrome P450; H(P)AEA, lipoxygenase products of anandamide; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; INDO, indomethacin; LOX, lipoxygenase; MethAEA, methanandamide; Mico, miconazole; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; PMSF, phenylmethylsulphonyl fluoride; TXA₂, thromboxan A₂. **b**, Effects of the blockers of AA metabolism on the elevation of $[Ca^{2+}]_i$ induced by 10 μ M AA in TRPV4-expressing (filled bars) and non-transfected cells (open bars). **c**, Effect of the same blockers on the basal $[Ca^{2+}]_i$ level. **d**, 4α PDD (1 μ M) still induces an increase in $[Ca^{2+}]_i$ in the presence of cytochrome P450 blockers, excluding a direct effect on the TRPV4 channel (all blockers were applied at a concentration of 10 μ M).

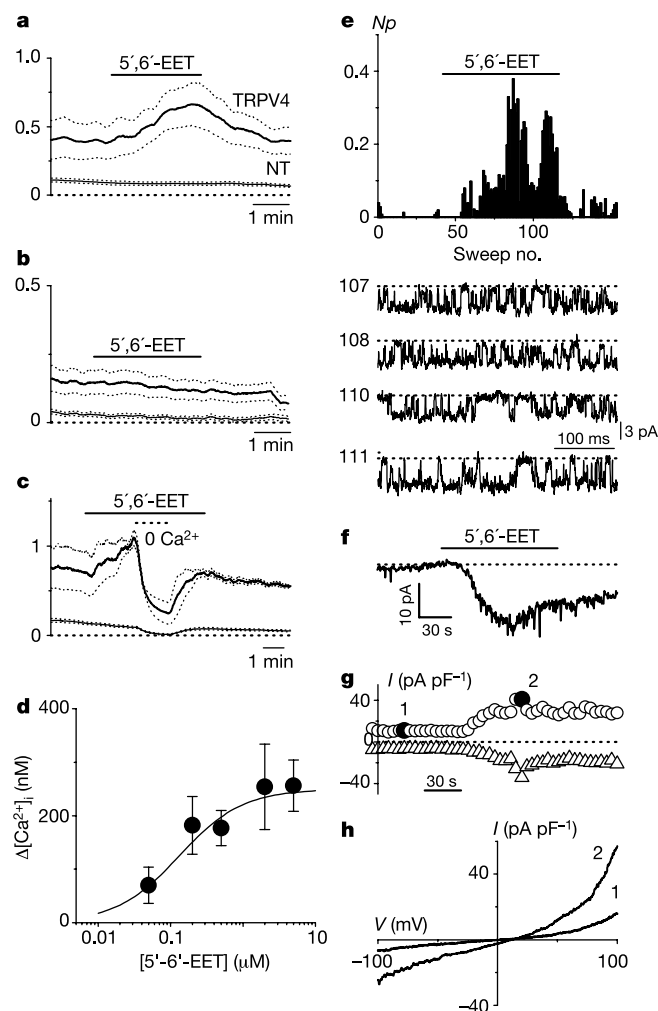


Figure 4 Activation of TRPV4 by EETs. **a**, **b**, Effect of 500 nM 5',6'-EET on $[Ca^{2+}]_i$ in the presence (**a**) and absence (**b**) of extracellular Ca^{2+} . **c**, Effect of the removal of extracellular Ca^{2+} (at the indicated time) on the response to 5',6'-EET (thick lines, TRPV4-transfected; thin lines, non-transfected). **d**, Dose-response curve for the increase in $[Ca^{2+}]_i$ by 5',6'-EET (Hill coefficient is 1). The concentration giving half-maximal response is 130 ± 86 nM. **e**, Effect of 5',6'-EET (5 μ M) applied to inside-out patches on average current (upper panel) from single-channel current traces shown in the bottom panel (holding potential -60 mV). **f**, Whole-cell inward current induced by 5',6'-EET at a holding potential of 0 mV (5 μ M, TRPV4-transfected cell). **g**, Time course of whole-cell current at -80 and $+80$ mV (same cell as in **f**). **h**, Current-voltage relations at the times indicated in **g**.

TRPV4 is highly expressed in endothelial cells⁴, and we have shown previously that 4 α PDD and heat activate TRPV4 channels in these cells^{7,8}. To investigate whether 5',6'-EET activates TRPV4 channels in a native environment, we measured its effects on $[Ca^{2+}]_i$ and membrane currents in endothelial cells isolated from mouse aorta (Fig. 5). Application of 500 nM 5',6'-EET caused a robust increase in $[Ca^{2+}]_i$ (Fig. 5a), which was inhibited by the TRPV4 blocker ruthenium red (10 μ M; Fig. 5b) and absent from Ca^{2+} -free extracellular solution (data not shown). 5',6'-EET exerted its effect on $[Ca^{2+}]_i$ in the same concentration range as in TRPV4-transfected HEK cells (Fig. 5c). The 5',6'-EET-induced increase in $[Ca^{2+}]_i$ persisted in the presence of the opener of large-conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) NS1619 (20 μ M) and the BK_{Ca} blocker charybdotoxin (100 nM), and at elevated extracellular K^+ concentrations (data not shown). We can therefore exclude the possibility that 5',6'-EET increases $[Ca^{2+}]_i$ solely by its effect on the driving force for Ca^{2+} as a consequence of the activation of BK_{Ca} channels. Application of 5',6'-EET (3 μ M) to the intracellular side of inside-out patches caused activation of single channels (Fig. 5d) with a conductance (52 ± 7 pS; $n = 3$) similar to that observed in TRPV4-expressing cells. In whole-cell recordings, extracellular

application of 10 μ M 5',6'-EET activated a moderately outwardly rectifying current (10 ± 5 pA pF⁻¹ at -80 mV) that reversed at 19 ± 6 mV ($n = 6$; Fig. 5e, f), which is consistent with the properties of TRPV4. These data indicate strongly that 5',6'-EET induces Ca^{2+} influx in vascular endothelial cells through activation of endogenous TRPV4 channels.

The endocannabinoids AEA and 2-AG as well as the EETs are well-known vasorelaxants¹⁷. The vasorelaxant responses to AEA have been attributed to its stimulatory effect on TRPV1 in perivascular nerve endings¹⁸, whereas EETs are considered to be potential endothelium-derived hyperpolarizing factors (EDHFs) because of their activating effect on smooth muscle Ca^{2+} -activated K^+ channels¹⁹. However, it has been reported that AEA and EETs also exert effects on vascular tone independently of their actions on TRPV1 or Ca^{2+} -activated K^+ channels¹². Our present results identify TRPV4 as a potential molecular target for these vasorelaxants.

Our data identify endocannabinoids and arachidonic acid as endogenous chemical activators of TRPV4, acting through the cytochrome-450-dependent formation of 5',6'-EET. Ca^{2+} entry induced by EETs has been reported previously in different cell types, including endothelial cells^{20–22}. However, here we have identified TRPV4 molecularly as a Ca^{2+} -permeable channel activated by EETs. Interestingly, TRPV4 is also expressed in parts of the brain such as the hypothalamus, and might therefore contribute to the psychoactive effects of endocannabinoids²³. TRPV4 encompasses in a single molecule the functions of a cell volume and temperature sensor and acts as an ionotropic receptor for 5',6'-EET, providing a striking example of the gating promiscuity of TRP channels. □

Methods

Cell culture

Human embryonic kidney (HEK-293) cells were grown in DMEM medium containing 10% (v/v) human serum, 2 mM L-glutamine, 2 U ml⁻¹ penicillin and 2 mg ml⁻¹ streptomycin at 37 °C in a humidity-controlled incubator with 10% CO₂. The 'primary explant' technique was used to obtain freshly isolated endothelial cells from mouse aorta, as described previously^{24,25}.

Transient expression of mTRP12

We used the bicistronic expression plasmid pdiTRP12, which carries the entire protein-coding region for TRPV4 (mouse, mTRP12) and the green fluorescent protein for transient transfection⁴.

Solutions

For electrophysiological measurements, the standard extracellular solution contained (in mM): 150 NaCl, 6 CsCl, 1 MgCl₂, 1.5 or 5 CaCl₂, 10 glucose, 10 Na-HEPES pH 7.4. When indicated, a high- Ca^{2+} solution was used, containing (in mM): 100 NaCl, 6 CsCl, 1 MgCl₂, 30 CaCl₂, 10 glucose, 10 Na-HEPES pH 7.4. The pipette solution was composed of (in mM): 20 CsCl, 100 caesium aspartate, 1 MgCl₂, 4 Na₂ATP, 0.08 CaCl₂, 10 BAPTA, 10 HEPES, pH adjusted to 7.2 with CsOH. For single-channel measurements, the pipette solution contained (in mM): 150 NaCl, 1 MgCl₂, 10 Na-HEPES pH 7.4. The bath solution was 150 KCl, 5 MgCl₂, 10 HEPES, 10 glucose (pH adjusted to 7.4 with KOH) for cell-attached patches, and 130 CsCl, 1 MgCl₂, 1 Na₂ATP, 0.034 CaCl₂, 5 EGTA, 10 HEPES for inside-out patches (pH adjusted to 7.2 with CsOH). 4 α PDD (Sigma) was used at a concentration of 1 μ M. AEA, 2-AG, AA and methanandamide (all from Sigma) were all used at a concentration of 10 μ M. For FAAH inhibition, the cells were incubated with phenylmethylsulphonyl fluoride (100 μ M; Sigma) for 30 min at 37 °C, before the addition of AEA²⁶. Nordihydroguaiaretic acid, indomethacin, ETYA, miconazole (all from Sigma), 17-ODYA (ICN Biomedicals), 20-hydroxyeicosatetraenoic acid and EETs (both from Biomol) were applied to the bath solution at the indicated concentrations. All experiments were performed at 22–25 °C.

Electrophysiological recordings

Whole-cell membrane currents were monitored with an EPC-9 (HEKA Elektronik, Lambrecht, Germany) using ruptured patches. The following ramp protocol was applied: voltage step to -100 mV for 20 ms followed by a 180-ms linear ramp to +100 mV; the sampling interval was 0.1 ms. This protocol was repeated every 5 s from a holding potential of 0 mV. Single-channel currents were sampled at 200- and 500- μ s intervals and filtered at 2 and 1 kHz for HEK-293 and endothelial cells, respectively. The interval between the traces was 0.67 s. The responses to all ligands were slow despite the use of a fast perfusion system.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured with a monochromator-based imaging system consisting of a Polychrome IV monochromator (Till Photonics, Martinsried, Germany) and a Roper Scientific charge-coupled device camera connected to an Axiovert 200M inverted microscope (Zeiss, Göttingen, Germany). Monochromator and camera were controlled by

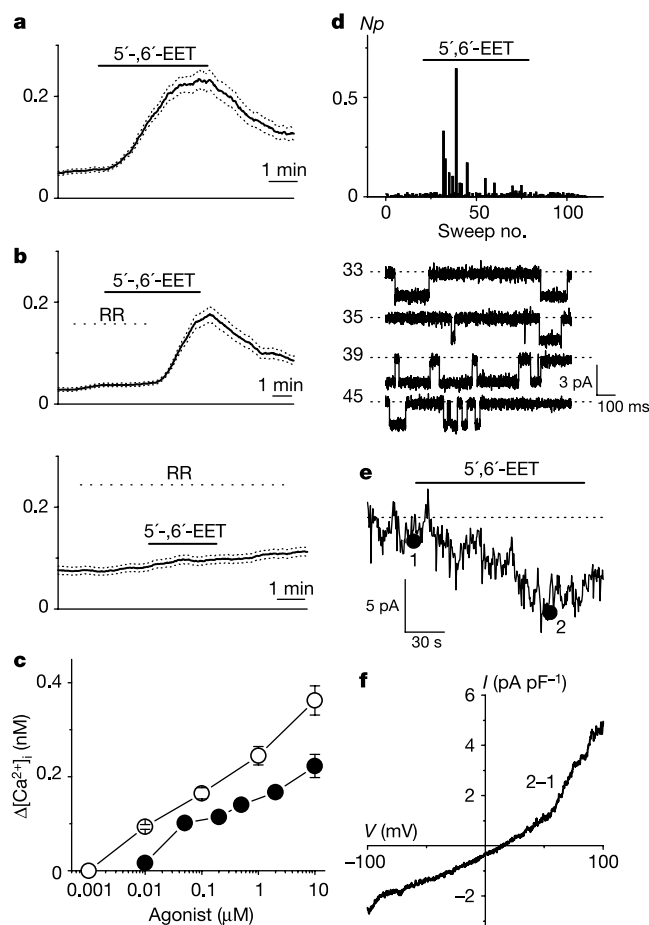


Figure 5 5',6'-EET-induced increase of $[Ca^{2+}]_i$ and activation of a TRPV4-like current in native endothelial cells from mouse aorta. **a**, $[Ca^{2+}]_i$ increase induced by 500 nM 5',6'-EET. **b**, Inhibition of the effect of 5',6'-EET by 10 μ M ruthenium red (RR). **c**, Dose-response relation for 5',6'-EET (filled circles) and 4 α PDD (open circles). Results are means $\pm$ s.e.m. **d**, Single-channel activation in an inside-out patch by 3 μ M 5',6'-EET. **e**, Whole-cell inward current induced by 5',6'-EET (5 μ M) at a holding potential of 0 mV. **f**, Difference between currents recorded at the times indicated in **e**, representing the current-voltage relation of the 5',6'-EET-activated current.

Metafluor software (West Chester, Pennsylvania, USA). Cells were loaded with Fura-2 by incubating them in a standard extracellular solution containing 2 μ M Fura-2 acetoxymethyl ester. Fluorescence was measured during alternative excitation at 340 and 380 nm and corrected for the individual background fluorescence. Absolute $[Ca^{2+}]_i$ values were calculated from the ratio, R , of the fluorescence signals at both wavelengths, as described previously²⁷. Ca^{2+} measurements are from 25–40 cells from at least five independent measurements.

Data analysis

Pooled data are given as means $\pm$ s.e.m. for n cells. Student's paired t -test was used to test for significant differences between non-transfected and TRPV4-transfected cells (single asterisk indicates $P < 0.05$; double asterisk $P < 0.01$).

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The expression domain of *PHANTASTICA* determines leaflet placement in compound leaves

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Diverse leaf forms in nature can be categorized as simple or compound. Simple leaves, such as those of petunia, have a single unit of blade, whereas compound leaves, such as those of tomato, have several units of blades called leaflets. Compound leaves can be pinnate, with leaflets arranged in succession on a rachis, or palmate, with leaflets clustered together at the leaf tip. The mechanisms that generate these various leaf forms are largely unknown. The upper (adaxial) surface is usually different from the bottom (abaxial) surface in both simple and compound leaves. In species with simple leaves, the specification of adaxial and abaxial cells is important for formation of the leaf blade^{1,2}, and the MYB transcription factor gene *PHANTASTICA* (*PHAN*) is involved in maintaining the leaf adaxial (upper) domain^{3,4}. Here we show that downregulation of *PHAN* is sufficient to reduce the adaxial domain of leaf primordia and to change pinnate compound leaves into palmate compound leaves. Furthermore, this mechanism seems to be shared among compound leaves that arose independently.

To determine how the adaxial domain affects compound leaf morphology (Fig. 1a–c) and how *PHAN* is involved in this processes, we modulated expression of the tomato *PHAN* orthologue (*LePHAN*) by expressing antisense *LePHAN* RNA (*antiLePHAN*) under the control of the CaMV 35S promoter. We found that 59 of 66 independent transgenic lines showed cup-shaped or needle-like leaves (Fig. 1e, f) that resembled the *phan* and *asl* mutant phenotypes^{2–4}. In addition, 43 lines showed palmate compound leaves (Fig. 1g–i and Supplementary Fig. 1a–d) instead of the pinnate compound leaves seen in wild-type tomato plants (Fig. 1d).

The petiole was radialized in palmate compound or needle-like *antiLePHAN* leaves (Fig. 1k), whereas wild-type petioles showed distinct ab-adaxiality (Fig. 1j) and produced leaflets in succession (Fig. 1l). Palmate compound leaves produced on *antiLePHAN* plants were peltate and formed a leaf blade (Fig. 1m) or leaflets (Fig. 1n) around the whole circumference of the petiole, including on the adaxial face. *LePHAN* RNA was detected in the leaf and stem vascular traces, and along the whole adaxial face of the P₃ and P₄ leaf primordium in wild-type tomato plants that produced pinnate compound leaves (Fig. 1o). Contrary to one report⁵ and consistent with another⁶, *LePHAN* RNA was also detected in the shoot apical meristem (Fig. 1o). *antiLePHAN* plants had reduced *LePHAN* expression overall, owing to antisense suppression. The expression domain of *LePHAN* in *antiLePHAN* transgenic plants was reduced to the distal region of the leaf primordium in plants with cup-shaped or palmate compound leaves (Fig. 1p, asterisk, and Supplementary Fig. 1e), and no *LePHAN* expression was seen in the leaf primordia of plants producing needle-like leaves (Fig. 1q).

Immunolocalization with a polyclonal antibody against ROUGH SHEATH2 (RS2), the maize orthologue of *PHAN*, confirmed that *PHAN* protein accumulation closely mirrored the pattern of RNA expression (Fig. 1r–t and Supplementary Fig. 1g–

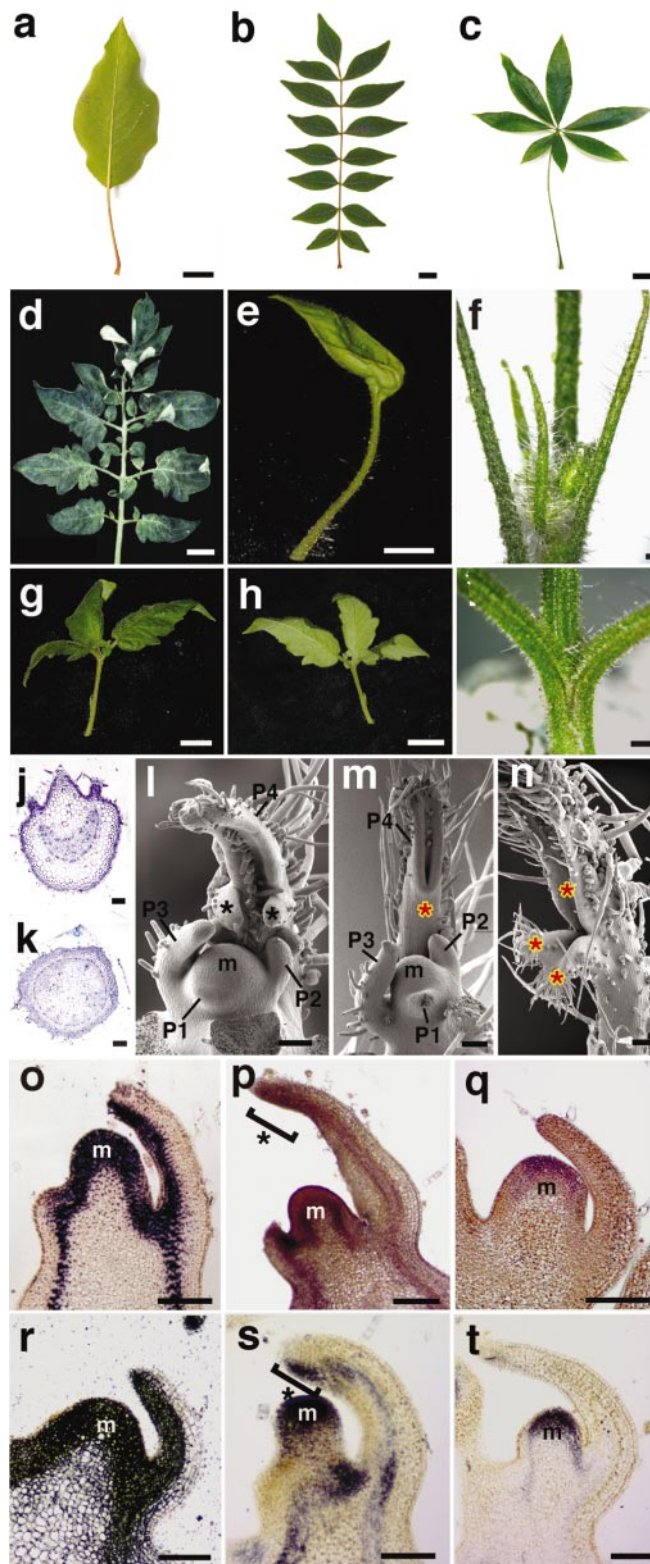


Figure 1 Leaf morphology. **a**, A simple leaf from *Malus domestica* (apple). **b**, A pinnate compound leaf from *Juglans regia*. **c**, A palmate compound leaf from *Pachira aquatica*. **d–t**, *LePHAN* expression domain and final leaf morphology in tomato. **d**, Wild-type tomato leaf. **e–i**, *antiLePHAN* transgenic plants produce cup-shaped (**e**), needle-like (**f**) and palmate (**g–i**) leaves. **i**, Close-up of the junction between the leaflets shown in **g**. **j, k**, Section of wild-type (**j**) and *antiLePHAN* (**k**) petiole. **l–n**, Scanning electron micrographs show that, unlike the wild type (**l**), the leaf blade (**m**) and leaflets (**n**) form in a peltate

arrangement in the cup-shaped or palmate leaves of *antiLePHAN* plants (red asterisks). **o–q**, *LePHAN* mRNA accumulates in the wild-type tomato shoot (**o**), and *antiLePHAN* transgenic plants produce cup-shaped or palmate (**p**) and needle-like (**q**) leaves. **r–t**, Pattern of *LePHAN* protein accumulation in the wild-type tomato shoot (**r**), and *antiLePHAN* transgenic plants produce cup-shaped or palmate (**s**) and needle-like (**t**) leaves. m, meristem; P1–P4, plastochron 1 to plastochron 4 leaves. Scale bars, 1 cm (**a–e, g, h**); 1 mm (**f, i**); 20 μ m (**j, k**); 100 μ m (**l–t**).

n). Together, these results suggest that there is a reduction in the leaf adaxial domain in *antiLePHAN* tomato plants. In addition, this transgene-induced reduction in the expression of *PHAN* often accompanied restriction of the adaxial domain to the distal end of the leaf primordium and resulted in the production of peltately palmate leaves in *antiLePHAN* plants.

To determine whether naturally occurring palmate compound leaves show a restriction of the adaxial domain to the distal part of the leaf primordium, we examined pinnate and palmate leaves from various taxa with compound leaves. A distinct adaxial domain was present in the petiole and rachis regions of pinnate compound leaves (such as *Fraxinus americana*; Fig. 2a, b). Unexpectedly, not all

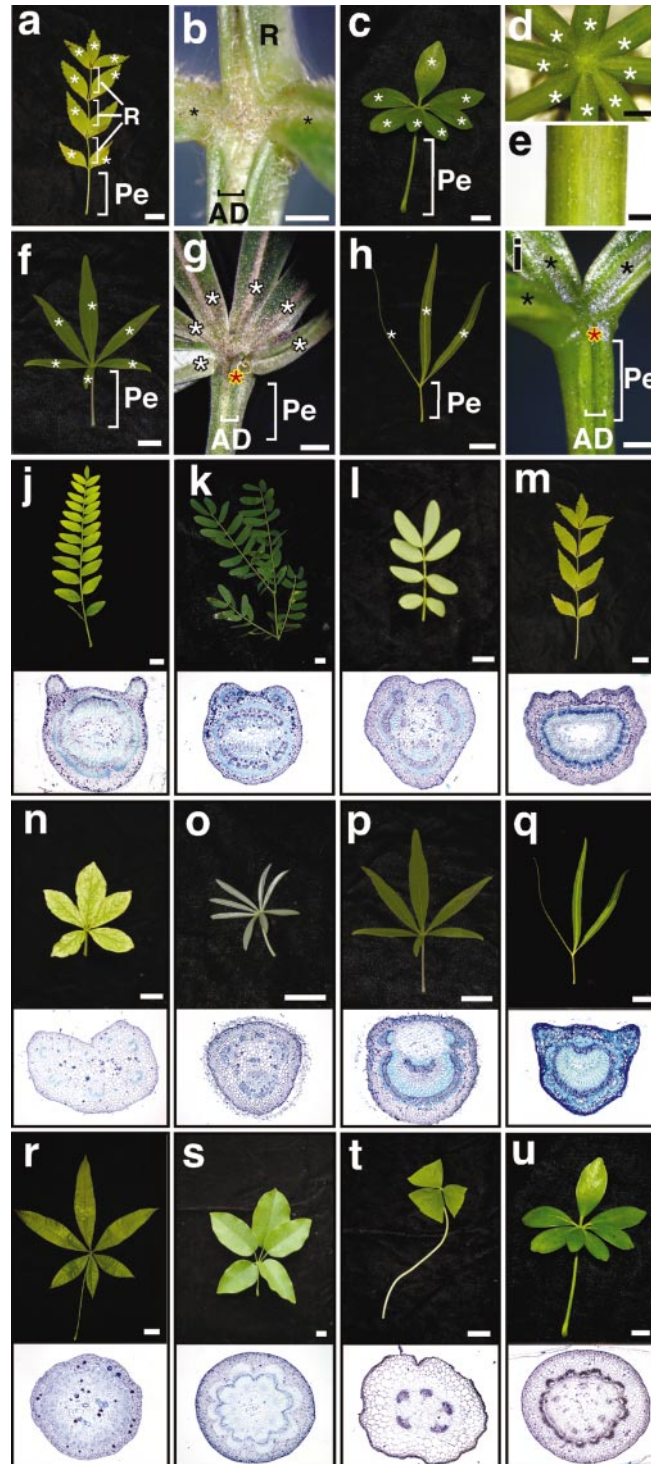


Figure 2 Adaxial domain in compound leaves from various plant species. **a, b**, Pinnate compound leaf from *F. americana*. **c–e**, Peltately palmate compound leaf from *S. actinophylla*, showing leaflets (**d**) and petiole (**e**). **f–i**, Non-peltately palmate leaves from *V. cannabinifolia* (**f, g**) and *R. lancea* (**h, i**). **j–m**, Pinnate compound leaves from *A. hindisii* (**j**) *L. coccinea* (**k**), *S. gaudichaudii* (**l**) and *F. americana* (**m**). **n–q**, Non-peltately palmate

leaves from *D. pentaphylla* (**n**), *L. albiifrons* (**o**), *V. cannabinifolia* (**p**) and *R. lancea* (**q**). **r–u**, Peltately palmate compound leaves from *P. aquatica* (**r**), *A. pentaphylla* (**s**), *O. regnellii* (**t**) and *S. actinophylla* (**u**). Asterisks, leaflets; AD, adaxial domain; Pe, petiole; R, rachis. Scale bars, 1 cm (**a, c, f, h, j–u**); 2 mm (**b, d, e, g, i**).

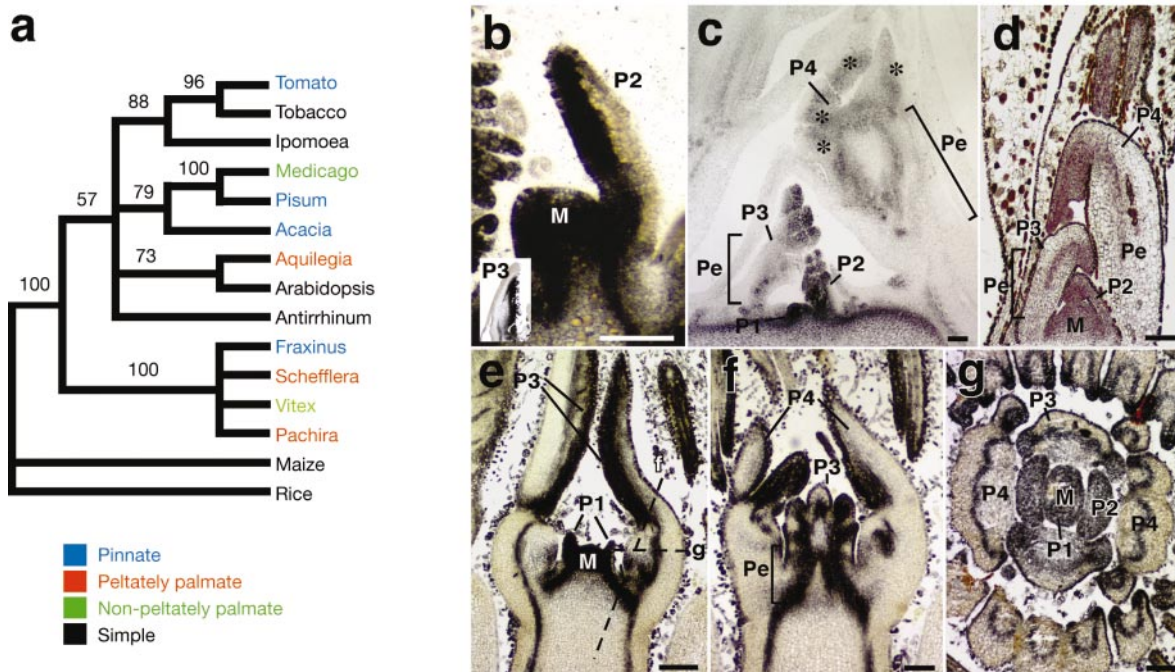


Figure 3 PHAN and compound leaf development. **a**, Phylogenetic relationships of PHAN orthologues based on parsimony analysis of amino acid sequences. Shown are bootstrap values from 1,000 replicates. **b–g**, PHAN expression domain and final morphology of the compound leaf. **b**, Pinnate compound leaves from *A. hindisii*. **c, d**, Peltately palmate leaves from *S. actinophylla* (**c**) and *O. regnellii*. (**d**).

e–g, Non-peltately palmate compound leaves from *V. cannabifolia*, showing longitudinal (**e**), sagittal (**f**) and transverse (**g**) sections. M, meristem; P1–P4, plastochron 1 to plastochron 4 leaves; Pe, petiole. Broken lines in **e** indicate the section planes in **f** and **g**. Scale bars, 100 μ m (**b–g**).

palmate leaves were similar in architecture. Certain leaves (non-peltately palmate compound) had a well-defined adaxial domain throughout the petiole. Leaflets were clustered at the tip of the petiole, but no leaflets were formed in the region where the adaxial domain existed at the tip of the petiole (Fig. 2f–i, red asterisk).

Sections showed distinct ab-adaxial symmetry in the petioles of pinnate (*Acacia hindisii*, *Senna gaudichaudii*, *Leea coccinea* and *F. americana*; Fig. 2j–m) or non-peltately palmate (*Dioscorea pentaphylla*, *Lupinus albifrons*, *Vitex cannabifolia* and *Rhus lancea*; Fig. 2n–q) compound leaves; however, peltately palmate compound leaves produced leaflets all around the tip of the petiole (such as *Schefflera*, Fig. 2c–e). The petioles of peltately palmate compound leaves (such as *Pachira aquatica*, *Akebia pentaphylla*, *Oxalis regnellii* and *Schefflera actinophylla*; Fig. 2r–u) were radially symmetrical with vascular bundles arranged in a ring, consistent with complete abaxialization of this region. In a survey of 25 angiosperm families, 289 species with pinnate compound and 153 species with non-peltately palmate compound leaves showed an adaxial domain extending from the tip of the leaf to the bottom. By contrast, in 56 different species with peltately palmate compound leaves, leaflets were produced all around the tip of petiole and the petiole lacked an adaxial domain (Supplementary Fig. 2a). This suggests that absence of the adaxial domain in the proximal region of petiole (petiole abaxialization) is important for generating peltately palmate compound leaves.

Fifteen PHAN orthologues from monocot and dicot species showed high conservation of amino acid sequence (Supplementary Fig. 2b, c). The PHAN orthologues always formed a monophyletic clade, which was distinct from all other known MYB proteins (data not shown). In phylogenetic analyses, the PHAN DNA and protein sequences were not grouped on the basis of final leaf morphology (Fig. 3a); rather, the gene tree was congruent with the known species phylogeny. In addition, no common amino acid residues specific to pinnate or palmate compound leaves were seen, suggesting that alterations in the coding region of PHAN did not have a role in the

evolution of leaf morphology. Thus, it is more likely that a change in the PHAN expression domain was crucial in the evolution of compound leaf forms and that these PHAN coding regions have maintained a conserved function to establish the adaxial domain in the leaf primordium.

The pattern of PHAN expression in diverse species with compound leaves (*S. actinophylla*, *F. americana*, *A. hindisii*, *V. cannabifolia*, *Dizygotheca elegantissima*, *O. regnellii*, *Koeleria paniculata*, *Aquilegia formosa* and *P. aquatica*) was determined by *in situ* polymerase chain reaction with reverse transcription (RT–PCR) and also by immunolocalization using the polyclonal antibody against RS2. All species examined showed PHAN expression in the shoot apical meristem, stem and leaf vascular traces. PHAN was expressed along the whole adaxial face of the P₂–P₄ leaf primordium of pinnate compound-leaved species such as *A. hindisii* (Fig. 3b) and *F. americana* (Supplementary Fig. 3a), resembling the pattern of LePHAN expression in wild-type tomato plants (Fig. 1o, r). By contrast, PHAN expression was confined to the distal region of the leaf primordium in peltately palmate compound-leaved species such as *S. actinophylla* (Fig. 3c and Supplementary Fig. 3q–t), *O. regnellii* (Fig. 3d and Supplementary Fig. 3b–e), *D. elegantissima* (Supplementary Fig. 3f–h) and *P. aquatica* (Supplementary Fig. 3i–p), and no PHAN expression was detected in the radial proximal region of the leaf primordium (other than in the vascular traces).

These data indicate that the PHAN expression domain sets up the adaxial domain, which is important for determining final morphology of the compound leaf. Expression of PHAN along the whole adaxial face of leaf primordia correlates perfectly with the formation of pinnate compound leaves, whereas confining PHAN expression to only the distal region of leaf primordia results in peltately palmate compound leaves (Fig. 4a). We propose that the boundary between adaxial and abaxial domains is required not only for blade formation but also for leaflet formation. In peltately palmate compound leaves, leaflets arise in a whorl in the distal

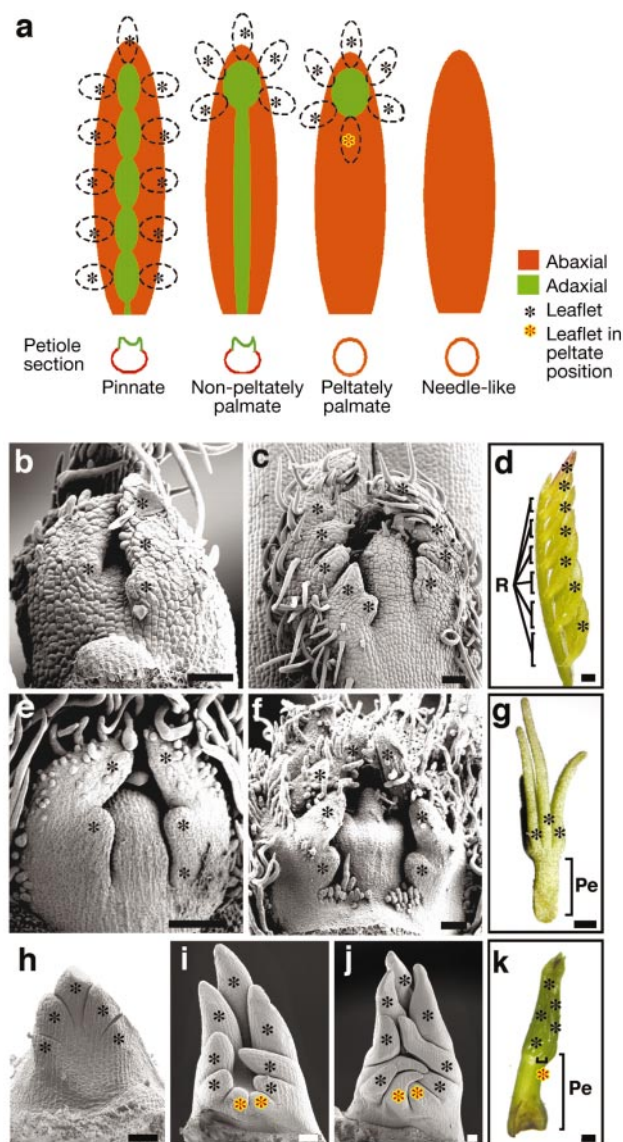


Figure 4 Compound leaf development in pinnate, peltately palmate and non-peltately palmate leaves. **a**, Adaxial (or PHAN expression) domain and final compound leaf morphology. **b, c, e, f**, Scanning electron micrographs of pinnate (*Acacia*, **b, c**) and non-peltately palmate leaves (*Vitex*, **e, f**) in the early stages of development. **d, g**, Pinnate compound (*Acacia*, **d**) and non-peltately palmate leaves (*Vitex*, **g**) in later developmental stages. **h–k**, Peltately palmate leaves (*D. elegantissima*) form leaflets in the peltate position. Scanning electron micrographs of the adaxial view of developing leaves are shown in **h–j**. Pe, petiole; R, rachis. Scale bars, 50 μ m (**b, c, e, f, h–j**); 500 μ m (**d, g, k**).

region of the leaf primordium because an adaxial domain is present only in this region (Fig. 4a).

PHAN was expressed along the adaxial face of the leaf primordium (P_3 and P_4) in *Vitex* with non-peltately palmate compound leaves (Fig. 3e–g). This expression pattern resembled that of pinnate compound leaves; however, the final leaf morphology of non-peltately palmate compound leaves was superficially similar to that of peltately palmate compound leaves. Scanning electron microscopy showed that leaflet development at early stages was almost identical in pinnate (Fig. 4b, c) and non-peltately palmate (Fig. 4e, f) compound leaves; however, final leaf morphology was determined by secondary morphogenesis later in development.

In pinnate compound leaves, the rachis region between leaflets

elongates as the leaf primordium grows (Fig. 4d). In non-peltately palmate compound leaves, only the basal region of the petiole elongates (Fig. 4g). By contrast, peltately palmate compound leaves develop leaflets on the adaxial side of the primordium (Fig. 4h–k, red asterisks). This suggests that non-peltately palmate leaves are a developmental variation of pinnate compound leaves and are morphogenetically distinct from peltately palmate leaves. We also propose that leaflet formation requires sufficient expression of *PHAN*. In support of this idea, the rachis in pinnate compound leaves and the basal region of the petiole in non-peltately palmate leaves have reduced expression of *PHAN*, and leaflets are not generated in these regions (Fig. 3g and Supplementary Fig. 1g).

The species in which *PHAN* expression was examined represent at least five independent occurrences of compound leaves, covering distantly related eudicots: the Ranunculales (*Aquilegia*) group, the Fabales (*Acacia*)/Malvales (*Pachira*)/Sapindales (*Koeleruteria*) group, the Oxalidales (*Oxalis*) group, the Solanales (tomato)/Lamiales (*Fraxinus* and *Vitex*) group and the Apiales (*Schefflera* and *Dizygotheca*) group^{7,8}. These expression patterns suggest that non-homologous compound leaves use the same mechanism (spatial modulation of the adaxial or *PHAN* expression domain) to control the final morphology of compound leaves. Several examples support the idea that changes in the regulation of a gene may be more important for morphological evolution in plants and animals^{7–12}.

Throughout leaf evolutionary history, changes in the *PHAN* expression domain seem to have been important for generating different leaf forms. In this study, the correlation between the extent of the *PHAN* expression domain and the adaxial domain of the leaf primordium indicates that *PHAN* orthologues maintained a conserved function to establish adaxial domains. While several genes may be involved in restricting the adaxial domain to the tip of the leaf in peltately palmate compound leaves, the *antiLePHAN* (tomato), *phan* (*Antirrhinum*) and *as1* (*Arabidopsis*) phenotypes indicate that *PHAN* may be a principal regulator of this process^{3,4,13}. Although maize plants mutated at the *RS2* locus do not show reduced adaxial features in the leaf^{14,15}, this may be due to inherent developmental differences between grass and dicot leaves. Compound-leaved species from independent origins showed *PHAN* expression in leaf primordia and leaflet primordia, which resembles the pattern of *PHAN* expression reported in tomato⁶. The control of leaf morphology by regulating *PHAN* expression has been reused over time, suggesting that there may be limited ways to alter compound leaf morphogenesis. It will be interesting to explore how *PHAN* expression is regulated in species with divergent leaf forms and whether this regulatory mechanism is conserved in non-homologous compound leaves. □

Methods

Tomato transgenic plants

The *antiLePHAN* construct was cloned into the pBIBKAN plasmid and introduced into tomato (*Lycopersicon esculentum* cv. VF36) by *Agrobacterium tumefaciens* LBA4404 as described¹⁶. We obtained 66 independent transformants.

In situ RT–PCR

In situ RT–PCR was done in tomato, *S. actinophylla*, *F. americana*, *A. hindisii* and *V. cannabifolia* as described¹⁷ by using the following primers: *LePHAN1*, 5′-ACGAGCAGCG TCTTGTATACAACTAC-3′; *LePHAN2*, 5′-CCCTTCGTCTAAATCCTTGCAGC-3′; *Fraxinus1*, 5′-ACAGCTACAGAAATCGCATAATAGCCGCC-3′; *Fraxinus2*, 5′-TCTTCC TTCTCAAGCTCTTTCAGTACTG-3′; *Acacia1*, 5′-TGTCCTCTCTTAACCTCTCTGC AACACTC-3′; *Acacia2*, 5′-GGCAAGTGGTGGGAAGTGTCAAAGAGAA-3′; *Schefflera1*, 5′-AAAACAGCTCAGGGACCTCCAAAAACCC-3′; *Schefflera2*, 5′-TGTC TTCTTCTCCAGTCTTTCAGTAC-3′; *Vitex1*, 5′-AGGCACTCCAGAAATCACC TGGATTAG-3′; *Vitex2*, 5′-TCCACCTCCTTGAATGCTGAATTAGCAC-3′.

Immunohistochemistry and microscopy

Immunolocalization studies were done in *S. actinophylla*, *F. americana*, *A. hindisii*, *V. cannabifolia*, *P. aquatica*, *D. elegantissima*, *K. paniculata*, *A. formosa* and *O. regnellii* as described¹⁸ with a polyclonal antibody against RS2. We cloned the non-MYB domain from RS2 (amino acids 133–370) into the pET-19b vector (Novagen), expressed the His₁₀ fusion

protein in *Escherichia coli* BL21 (DE3) and purified the RS2 fusion protein by Ni-NTA affinity chromatography (Qiagen). The fusion protein was separated by SDS-PAGE, and gel slices containing about 300 ng of protein were pulverized and used directly to immunize rabbits. The His₁₀-RS2 fusion protein was coupled to Affi-Gel 10 beads (Bio-Rad) to generate an affinity column. Polyclonal antiserum was loaded onto the affinity column (serum, 8 ml; bed volume, 2 ml) and the column was washed extensively with TBS (20 mM Tris-HCl (pH 7.4) and 0.15 M NaCl) and 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl and 0.2% Triton X-100. Antibodies were eluted in 0.2 M glycine-HCl (pH 2.0) and 0.15 M NaCl, and the collected fractions were immediately neutralized with 60 µl of 2 M Tris-HCl (pH 8.5). Each fraction was dialysed against TBS overnight at 4 °C. We determined wild-type and rs2 mutant apices. Scanning electron microscopy was done as described¹⁹.

Phylogenetic analysis

Nine PHAN orthologues were obtained from GenBank (accession numbers: rice, AB071600; Medicago, AF308453; tobacco, AJ006181; *Antirrhinum*, AJ005586; tomato, AF148934; maize, AF143447; *Arabidopsis*, NM_129319; pea, AF299140; *Ipomoea batatas*, BM878751). Additional PHAN orthologues from pinnate and palmate compound-leaved species (*A. formosa*, *V. cannabifolia*, *S. actinophylla*, *A. hindisii*, *P. aquatica* and *F. americana*) were cloned by PCR with the following degenerate primers, designed on the basis of available PHAN orthologue sequences: DePHAN1, 5'-CACGGNAACAARTGG AARAA-3'; DePHAN2, 5'-GCTTCRATYTCCTCCATYTT-3'. We aligned nucleotide and amino acid sequences by ClustalX (Supplementary Fig. 2c) and carried out parsimony analyses by PAUP4 and McClade.

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Correspondence and requests for materials should be addressed to N.S. (nrsinha@ucdavis.edu). The sequences are deposited in GenBank under accession codes AY180131, *A. formosa*; AY180132, *V. cannabifolia*; AY180133, *S. actinophylla*; AY180134, *A. hindisii*; AY180135, *P. aquatica*; AY180136, *F. americana*.

GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5

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Congenital heart defects (CHDs) are the most common developmental anomaly and are the leading non-infectious cause of mortality in newborns¹. Only one causative gene, *NKX2-5*, has been identified through genetic linkage analysis of pedigrees with non-syndromic CHDs^{2,3}. Here, we show that isolated cardiac septal defects in a large pedigree were linked to chromosome 8p22-23. A heterozygous G296S missense mutation of *GATA4*, a transcription factor essential for heart formation^{4–7}, was found in all available affected family members but not in any control individuals. This mutation resulted in diminished DNA-binding affinity and transcriptional activity of Gata4. Furthermore, the Gata4 mutation abrogated a physical interaction between Gata4 and TBX5, a T-box protein responsible for a subset of syndromic cardiac septal defects^{8,9}. Conversely, interaction of Gata4 and TBX5 was disrupted by specific human *TBX5* missense mutations that cause similar cardiac septal defects. In a second family, we identified a frame-shift mutation of *GATA4* (E359del) that was transcriptionally inactive and segregated with cardiac septal defects. These results implicate *GATA4* as a genetic cause of human cardiac septal defects, perhaps through its interaction with TBX5.

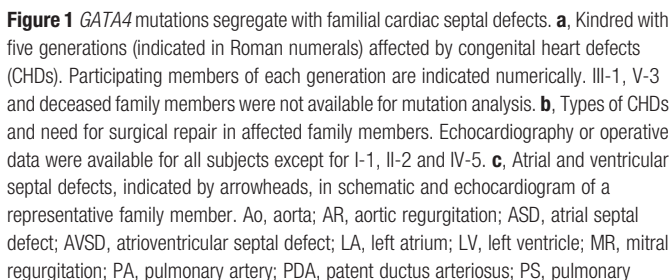
Division of a common cardiac atrium and ventricle into right- and left-sided chambers represents an essential evolutionary milestone in development of the four-chambered heart and is necessary for separation of oxygenated and deoxygenated blood. In humans, failure of atrial or ventricular septation accounts for nearly 50% of CHDs and requires open-heart surgery to restore normal circulation¹. Although cardiac septal defects (CSDs) are common, the precise molecular mechanisms for cardiac septal closure in humans remain to be elucidated. Mutations in *NKX2-5* have been identified in individuals with CSDs and conduction abnormalities, whereas individuals with Holt–Oram syndrome (HOS)—characterized by CSDs, conduction abnormalities and limb anomalies—have point mutations in *TBX5* (refs 2, 8, 9).

We identified a large kindred spanning five generations in which 16 individuals had CHDs (Fig. 1a). Detailed clinical evaluations were reviewed for all available family members, and demonstrated an autosomal dominant pattern of inheritance. All affected members had atrial septal defects. Eight individuals had additional forms of CHDs, including ventricular septal defects, atrioventricular septal defects, pulmonary valve thickening, or insufficiency of

Direct sequencing of *NKX2-5* and *TBX5* in this family failed to reveal mutations, suggesting an alternative genetic aetiology. We performed a genome-wide scan of all family members that revealed linkage of the CHD phenotype to a single locus on chromosome 8p22-23 (LOD score = 5.7, $\theta = 0$) between D8S264 and D8S1827, spanning approximately 30 cM (about 12.7 megabases). Examination of genes in this interval revealed the presence of *GATA4*, which encodes a zinc-finger transcription factor essential for cardiogenesis in flies, fish and mice⁴⁻⁷. Direct sequencing of *GATA4* in an affected family member identified a G-to-A transition of nucleotide 886 (Fig. 1d) that predicted a glycine to serine substitution at codon 296 (hG296S) (Fig. 2b). All affected individuals that were clinically evaluated had the hG296S mutation, suggesting complete penetrance of the disease phenotype (Fig. 1d). The mutant allele was not detected in unaffected family members nor in 3,000 unrelated individuals of diverse ethnicity, making it unlikely that hG296S represented a rare polymorphism. In the proband, we sequenced 100 additional regulatory genes essential for, or expressed during, cardiac development but failed to identify other linked mutations,

By direct sequencing of *GATA4*, we identified a second family with autosomal dominant transmission of atrial septal defects in which a mutation of *GATA4* (hE359del) was found in all available affected members spanning four generations (Fig. 1e, f; see also Supplementary Fig. 2). Similar to family A, neither the cardiac conduction system nor other organs were affected in this kindred. The hE359del mutation in this pedigree resulted in a frame shift past amino acid 359, thus severely altering the encoded GATA4 protein (Fig. 1g). A premature stop codon is predicted by the frame shift and may result in a truncated protein or nonsense-mediated decay of the transcript¹⁰. This nucleotide deletion was not found in unaffected family members or in 300 other individuals. The segregation of *GATA4* mutations with cardiac septal defects in two large unrelated families provides strong evidence that mutations in *GATA4* are the underlying cause of a subset of familial, non-syndromic cardiac septal defects. Consistent with this, deletion of the terminal end of chromosome 8p that contains *GATA4*, among many other genes, is characterized by cardiac septal defects¹¹.

GATA4 belongs to a family of transcription factors that binds a consensus HGATAR DNA motif and contains two class IV zinc-finger domains^{12–14}. Gata1, Gata2 and Gata3 are expressed predominantly in haematopoietic cells, and heterozygous mutations of *GATA1* and *GATA3* cause human blood disorders and organ malformations, respectively^{15,16}. In contrast, Gata4, Gata5 and



stenosis; RA, right atrium; RV, right ventricle; VSD, ventricular septal defect. **d**, Sequence chromatogram displaying G to A transition of nucleotide 886, generating a new *Pst*I restriction enzyme site in exon 3 of *GATA4* that resulted in the appearance of 234-bp and 161-bp fragments of a 395-bp PCR product in affected members of family A. **e**, Second pedigree affected by CHDs, as described in **f**. **g**, Sequencing revealed a single nucleotide deletion that was linked to disease and altered the *GATA4* sequence after amino acid 359, resulting in a premature stop codon. The wild type (WT) and predicted mutant (MT) protein sequences are shown. Two (II-2 and III-1) affected members were not available for the mutation study.

Gata6 are expressed in the developing heart and in several endodermal lineages, but have not been implicated in human disease^{14,17}. However, null mutations in the *Drosophila* Gata4 orthologue, *pannier*⁶, zebrafish *gata5* (ref. 7) or mouse *Gata4* (refs 4, 5) result in early defects in cardiogenesis. Although mice heterozygous for *Gata4* do not have obvious cardiac anomalies, further reduction in Gata4 dosage from a hypomorphic *Gata4* allele causes cardiac septal and other congenital heart defects (W. Pu and S. Izumo, personal communication).

The hG296S mutation affects a residue highly conserved across species and lies adjacent to the nuclear localization signal (NLS) and the carboxy-terminal zinc finger, whereas the hE359del mutation results in truncation of the last forty amino acids (Fig. 2a, b). We generated both mutations in the highly conserved mouse orthologue (mG295S or mE360del) and tested their abilities to activate transcription of downstream genes *in vitro*, using several Gata-dependent cardiac enhancers upstream of a luciferase reporter. When equivalent amounts of protein were expressed, mG295S displayed less transcriptional activation of the alpha myosin heavy chain (*Myhc*)¹⁸ and atrial natriuretic factor (*ANF*)¹⁹ enhancers

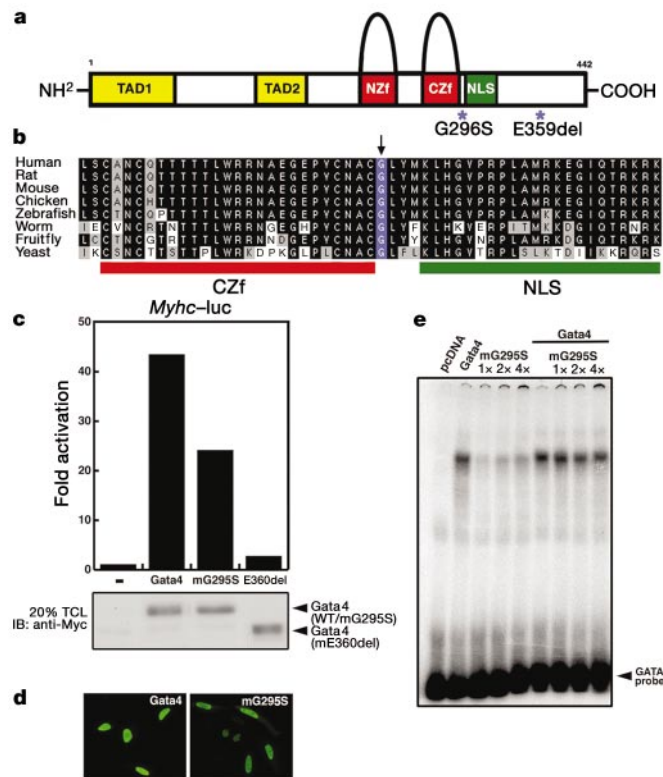


Figure 2 Functional deficits associated with Gata4 mutations. **a**, Schematic of GATA4 protein domains indicates transactivation domains (TAD), N-terminal zinc finger (NZf), C-terminal zinc finger (CZf) and nuclear localization signal (NLS). The location of hG296S and hE359del mutations is indicated. **b**, Cross-species alignment of amino acids in the region of the hG296S mutation. Conserved glycine residues are highlighted in blue with identical residues shaded in black; the CZf or NLS is indicated in red or green, respectively. **c**, Activation of *Myhc*-luciferase reporter in HeLa cells by Gata4 wild type (WT), mG295S or mE360del demonstrated decreased transactivation by mutant proteins, with protein levels indicated by immunoblot (IB) of total cell lysate (TCL). **d**, Nuclear localization of Myc-tagged Gata4 wild type or mG295S, represented in green, was similar when expressed in HeLa cells. **e**, Electromobility shift assay of ³²P-labelled GATA cis-element with Gata4 wild type or mG295S reveals decreased DNA-binding affinity of the mutant, even at four times concentration. The presence of increasing amounts of mutant protein did not affect the ability of wild-type protein to bind DNA.

compared to wild type, suggesting mildly reduced activity in this overexpression system (Fig. 2c and data not shown). In contrast, the truncated protein generated from recombinant mE360del was unable to activate transcription of either reporter (Fig. 2c). Although it cannot be determined whether transcripts encoding hE359del undergo nonsense-mediated decay *in vivo*, the inability of

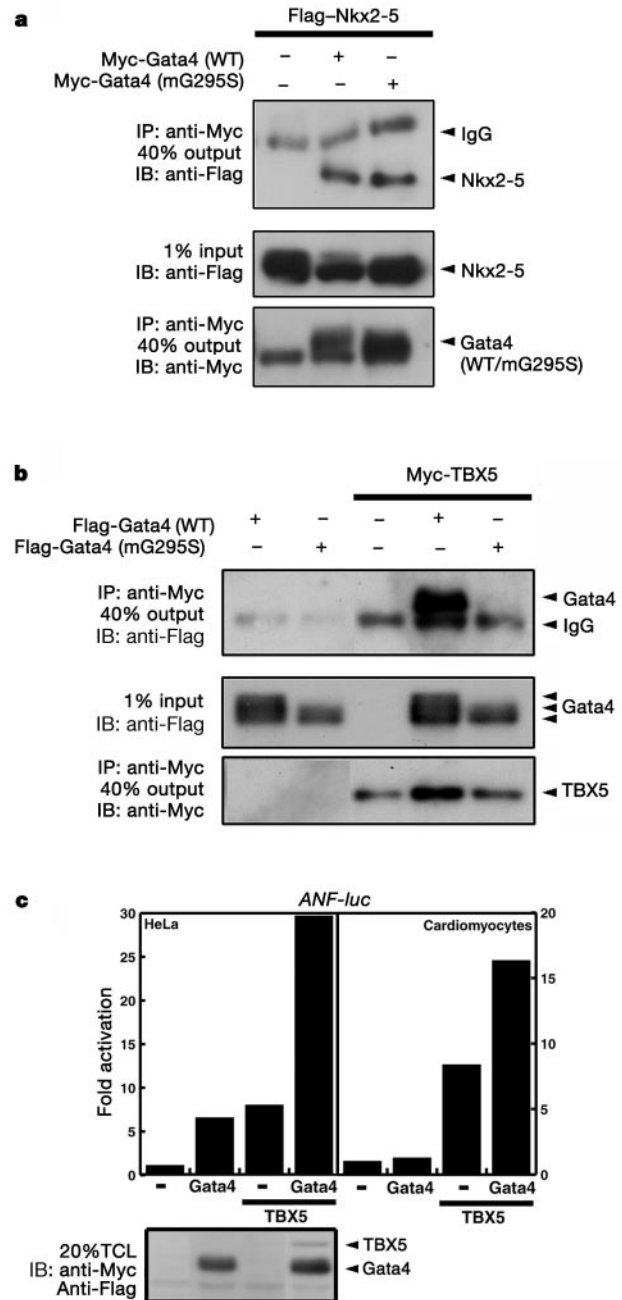


Figure 3 The Gata4 interaction with TBX5 is specifically disrupted by the Gata4 mG295S mutation. **a**, Immunoprecipitation (IP) with Myc-tagged wild-type or mutant Gata4 (mG295S) revealed an association of each with Flag-Nkx2-5 on immunoblot (IB) with anti-Flag antibody. IgG, immunoglobulin band. **b**, Co-immunoprecipitation of Myc-TBX5 demonstrated an association with Flag-Gata4 wild type but not mG295S. **c**, Luciferase (luc) activity directed by the *ANF* promoter in the presence of Gata4 and TBX5 in HeLa or neonatal rat ventricular cardiomyocytes displayed cooperative activation. Protein levels of Gata4 and TBX5 are indicated by IB. TCL, total cell lysate.

mE360del to activate transcription suggests that GATA4 is probably haploinsufficient.

Because mG295S functioned as a hypomorph, we investigated the mechanisms through which this mutation affected Gata4 function. Although mG295S is near the NLS, the protein localized to the nucleus in HeLa and COS1 cells, suggesting that the NLS was not disrupted (Fig. 2d and data not shown). mG295S is also immediately adjacent to the C-terminal zinc finger, which is essential for DNA-binding and interaction with co-factors²⁰ (Fig. 2a, b). In electromobility shift assays (EMSA) to test DNA-binding affinity, wild-type Gata4 efficiently retarded gel migration of a ³²P-labelled GATA *cis*-element corresponding to the *Hand2* enhancer²¹. In contrast, equivalent amounts of mG295S displayed limited affinity for the GATA element (Fig. 2e). With two- or fourfold excess of mG295S, we observed only weak DNA binding. When wild-type Gata4 was co-expressed in the presence of increasing amounts of mG295S, no alteration of wild-type DNA-binding affinity was detected (Fig. 2e). Together, these data suggest that the impaired DNA-binding of Gata4 mG295S may contribute to the reduced transcriptional activity of the mutant, although

forced overexpression of the hypomorphic protein in tissue culture may compensate for the decreased DNA-binding affinity.

The zinc-finger domains of Gata4 also mediate numerous protein-protein interactions that often dictate DNA-binding specificity and therefore subsets of target gene activation. Hence, we tested whether mG295S affected the ability of Gata4 to interact with the two other transcriptional regulators implicated in human cardiac septal formation; that is, NKX2-5 and TBX5. By immunoprecipitation, mG295S was able to interact with Nkx2-5 through the C-terminal zinc finger, similar to wild-type Gata4 (ref. 22), suggesting that this motif was intact (Fig. 3a). In addition, we discovered a new interaction between Gata4 and TBX5, as both wild-type proteins could immunoprecipitate with one another (Fig. 3b). However, mG295S could not interact with wild-type TBX5, suggesting specific disruption of the Gata4-TBX5 interaction by the Gata4 mutation (Fig. 3b).

To determine whether Gata4 cooperates with TBX5 to activate transcription, we co-transfected the *ANF* reporter with TBX5 and Gata4 in HeLa cells and primary cardiomyocytes. Similar to Tbx5-Nkx2-5 interactions^{23,24}, co-expression of TBX5 and Gata4 resulted in cooperative activation of the reporter (Fig. 3c). Consistent with this observation, when we co-expressed Gata4 and TBX5, we were unable to detect an increase in transactivation of a luciferase reporter downstream of six tandem GATA sites (data not shown). We were also unable to detect a tertiary complex of TBX5 and Gata4 with the GATA *cis*-element by EMSA (data not shown), suggesting that additive transactivation of the complex may require DNA-binding of both Gata4 and TBX5.

The similarity between cardiac septal defects observed in the setting of human *GATA4* mutations and human *TBX5* mutations is consistent with the Gata4-TBX5 complex described here. Human *NKX2-5* mutations also cause similar septal defects, and *NKX2-5* can physically interact with TBX5 (refs 23, 24). We therefore asked whether reported human *TBX5* mutations affect the ability of TBX5 to interact with GATA4 or NKX2-5. Six unique *TBX5* missense mutations from families that display HOS²⁵⁻²⁷ (Fig. 4a) were examined for their ability to interact with wild-type Gata4 or Nkx2-5. All *TBX5* mutants co-immunoprecipitated with Gata4 or Nkx2-5 except for the G80R and R237W mutants (Fig. 4b). In contrast, G80R and R237W were able to interact with several other cardiac transcription factors (I.S.K. and D.S., unpublished data), suggesting specific disruption of TBX5-Gata4/Nkx2-5 complexes. The reciprocal evidence that human *GATA4* and *TBX5* mutations affect interaction with one another in the setting of similar CHDs provides support that GATA4 and TBX5 may cooperate during cardiogenesis.

The combination of human genetics and biochemical analyses used here reveal a previously unrecognized genetic aetiology for CHDs and potential mechanisms through which certain cardiac defects may occur. The mutations in *GATA4* suggest that haploinsufficiency of *GATA4* in humans can result in the most common types of cardiac malformations and that GATA4 is essential for functional separation of the four cardiac chambers. In particular, the G296S mutation of GATA4 demonstrated not only an effect on DNA-binding affinity and transactivation of downstream targets, but also revealed an interaction between GATA4 and TBX5 that is disrupted in the setting of CHD. The similar effect of TBX5 mutations on the interaction with GATA4 and NKX2-5 raises the possibility that TBX5, NKX2-5 and GATA4 function in a complex to regulate a subset of genes required for cardiac septal formation. Whereas haploinsufficiency of *TBX5* has been linked to cardiac septal malformation in HOS, the disruption of a GATA4-TBX5 complex by some *TBX5* point mutations provides a potential mechanistic understanding of how disruption of combinatorial interactions of transcription factors can lead to specific birth defects. Broad screening for *GATA4* mutations in humans with heart disease may provide new avenues for understanding the

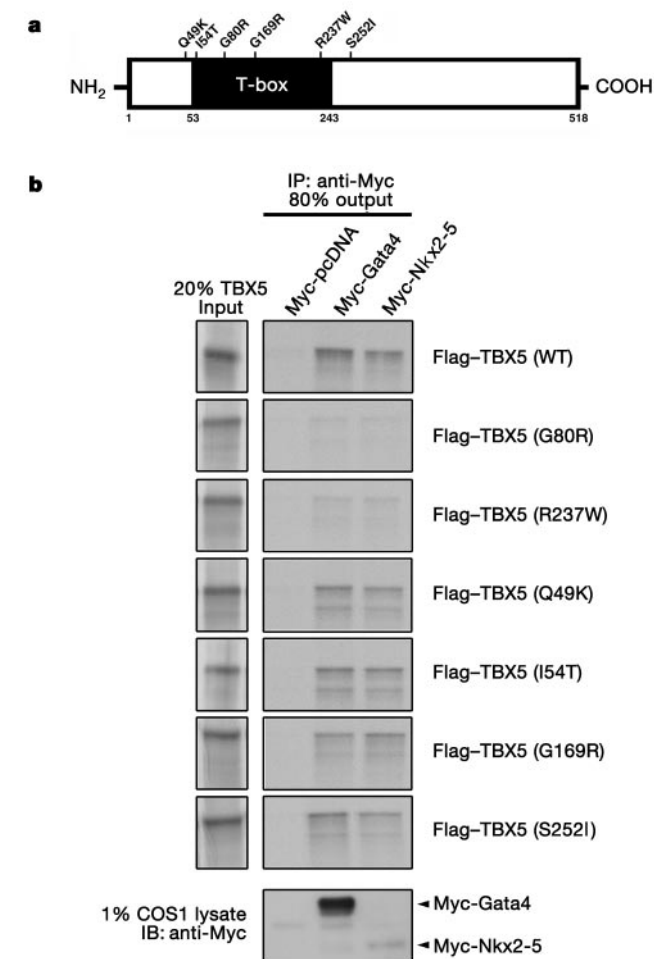


Figure 4 Disrupted interaction of human TBX5 mutant proteins with Gata4 and Nkx2-5. **a**, Schematic of mutations of *TBX5* in humans with Holt-Oram syndrome. **b**, All ³⁵S-labelled TBX5 mutants co-immunoprecipitated with Myc-Gata4 and Myc-Nkx2-5 with similar affinity to wild-type Flag-TBX5, except for the G80R and R237W mutants, when immunoprecipitated with an anti-Myc antibody and visualized by autoradiography. TBX5 input controls are shown on the left; immunoblot (IB) of Myc-tagged control proteins are shown at the bottom.

development of CHDs, leading to future therapeutic or preventive interventions. □

Methods

Clinical evaluation and DNA collection

CHD families were ascertained for genetic analyses at Children's Medical Center of Dallas, University of Texas Southwestern Medical Center (family A), and Tokyo Women's Medical University (family B). Clinical evaluations and genetic studies were performed in accordance with human subject guidelines after informed consent according to the protocol approved by the individual Institutional Review Boards. Family members were studied by history, physical examination, 12-lead electrocardiogram, and echocardiography. Medical records were reviewed of individuals who had died. Genomic DNA was extracted from peripheral lymphocytes for genetic analyses.

Genetic linkage analysis

A whole-genome linkage analysis was performed using 358 polymorphic DNA markers at about 11 cM intervals (Cooperative Human Linkage Center/Weber Human Screening Set Version 8, Research Genetics, Inc.). Markers were genotyped in all family members as previously described²⁸. Linkage analysis was performed using GENEHUNTER²⁹.

Identification of *GATA4* mutations

All exons of the *GATA4* gene were sequenced in both directions to search for mutations in a proband from both pedigrees. The third or fifth exon of the human *GATA4* gene was amplified from family A or B, respectively, by PCR (primers and conditions available on request). The 395-bp (exon 3) or 411-bp (exon 5) PCR product was sequenced in both directions. The 395-bp fragment was digested with *Pst*I, resulting in the generation of two bands of 234 bp and 161 bp in subjects harbouring the mutant G296S *GATA4* allele; the 411-bp fragment was digested with *Bse*RI resulting in 142-bp and 269-bp fragments in subjects without the E359del mutation.

Plasmid construction and site-directed mutagenesis

*Myhc-luc*¹⁸, *ANF-luc*¹⁹ and Flag-TBX5 (ref. 23) have been described previously; 6 × *GATA* site-luc was provided by S. R. Grant. For additional expression constructs, cassettes flanked by unique restriction sites were generated by PCR and cloned into pcDNA3.1-N-Myc and pcDNA3.1-N-Flag vectors (Invitrogen). Point mutations in *Gata4* or *TBX5* were introduced by generating two overlapping fragments by PCR and were verified by sequencing.

Luciferase assays

HeLa cells were transfected using Eugene 6 (Roche) with 300 ng reporter, 300 ng *Gata4*, 600 ng *Gata4* mG295S, 300 ng *Gata4* mE360del or 300 ng *TBX5*. Immunoblots were used to verify appropriate protein expression. mG295S was consistently expressed at lower levels than wild type, necessitating transfection of twofold greater plasmid to achieve similar protein levels. Neonatal rat ventricular cardiomyocytes were collected and transfected with 150 ng reporter, 200 ng *Gata4* and 400 ng *TBX5*, using Lipofectamine Plus reagent (Invitrogen). Luciferase activity was measured 48 h after transient transfection as previously described³⁰. At least three independent experiments were performed in duplicate, with representative data shown.

Immunocytochemistry

Forty-eight hours after transient transfection with Eugene 6 (Roche), HeLa and COS1 cells grown on slides were fixed with 3.7% formaldehyde/PBS, permeabilized with 0.1% TritonX-100/PBS, incubated with monoclonal anti-Myc antibody (Santa Cruz) and detected using anti-mouse conjugated-FITC antibody (Jackson ImmunoResearch).

Electrophoretic mobility shift assay

Annealed oligonucleotides (5'-TCGAGGTAATTAAC7GATAATGGTGC-3') with a GGG overhang representing the *GATA cis*-element upstream of *Hand2* (ref. 21) were labelled with [³²P]dCTP using Klenow enzyme and incubated with 2λ of wild-type and/or 2–8λ of mG295S *Gata4* synthesized using the TNT Quick Coupled Transcription/Translation System (Promega) in binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM MgCl₂, 0.5 mM DTT and 10% glycerol) for 20 min at room temperature and separated on a 6% polyacrylamide gel. [³²S]-labelled protein products were verified by SDS-PAGE.

Co-immunoprecipitation assays

COS1 cells were transiently transfected with Eugene 6 (Roche), collected after 48 h in lysis buffer (PBS, 1 mM EDTA, pH 8.0, 0.5% Triton X-100), incubated with polyclonal anti-Myc antibody (Santa Cruz) and protein A Sepharose beads (Amersham Pharmacia), immunoblotted with monoclonal anti-Myc (Santa Cruz) or anti-Flag M2 (Sigma) antibodies and detected by the AP-conjugated CDP-Star system (Perkin Elmer). For overexpression of *TBX5* point mutations, ³⁵S-labelled products were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega), incubated with COS1 lysates transfected with Myc-*Gata4* or Myc-Nkx2-5, immunoprecipitated with polyclonal anti-Myc, and visualized by autoradiography.

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The molecular nature of the zebrafish tail organizer

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Based on grafting experiments, Mangold and Spemann showed the dorsal blastopore lip of an amphibian gastrula to be able to induce a secondary body axis¹. The equivalent of this organizer region has been identified in different vertebrates including teleosts². However, whereas the graft can induce ectopic head and trunk, endogenous and ectopic axes fuse in the posterior part of the body^{3,4}, raising the question of whether a distinct organizer region is necessary for tail development. Here we reveal, by isochronic and heterochronic transplantation, the existence of a tail organizer deriving from the ventral margin of the zebrafish embryo, which is independent of the dorsal Spemann organizer. Loss-of-function experiments reveal that bone morphogenetic protein (BMP), Nodal and Wnt8 signalling pathways are required for tail development. Moreover, stimulation of naive cells by a combination of BMP, Nodal and Wnt8 mimics the tail-organizing activity of the ventral margin and induces surrounding tissues to become tail. In contrast to induction of the vertebrate head, known to result from the triple inhibition of BMP, Nodal and Wnt⁵, here we show that induction of the tail results from the triple stimulation of BMP, Nodal and Wnt8 signalling pathways.

At the beginning of gastrulation, marginal cells undergo differential convergence extension movements according to their position along the dorso-ventral axis. Whereas dorsal marginal cells involute and extend strongly towards the animal pole, the most ventral ones (called the “no convergence no extension zone”⁶) slip over the yolk^{6–8} (Fig. 1a). At the end of gastrulation, aggregation of marginal cells establishes the tail bud, which comprises cells deriving from the dorsal organizer as well as ventral marginal cells^{7,8} (Fig. 1b). Later in development, cells originating from the dorsal organizer form the chordo-neural hinge, which differentiates into notochord, hypochord and ventral neural tube (Fig. 1c, d), whereas the ventral gastrula margin is responsible for the formation of ventral and lateral parts of the tail bud that give non-axial tissues^{6,8} (Fig. 1c, e).

We investigated experimentally the contribution of these different subpopulations of cells to development of the tail. Surgical extirpation of the presumptive chordo-neural hinge at the end of gastrulation (Fig. 1f) gives individuals with wild-type morphology down to the level of the anus, which lack axial derivatives in their tail, whereas non-axial structures remain unaffected (Fig. 1g, h; see also Supplementary Fig. S1a–c). These tails are comparable with those of embryos ventralized through overexpression of BMPs⁹, which fail to form the embryonic shield—the zebrafish equivalent of the Spemann organizer—and in consequence, do not develop any axial structures (Fig. 1i; see also Supplementary Fig. S1d). Conversely, loss of ventral cell identity (by dorsalization of the embryo⁹ or by inhibition of Wnt8 signalling through Wnt8 morpholino knock down¹⁰ or through overexpression of a Wnt-specific inhibitor, Frzb^{11,12}) results in embryos devoid of non-axial structures of the tail (Fig. 1j, k; see also Supplementary Fig. S3c, j, k). In such individuals, in absence of non-axial tissues, axial structures develop and often coil on themselves. These various phenotypes reveal clearly that axial and non-axial tissues can develop independently of each other.

Formation of the tail bud at the end of gastrulation and its subsequent outgrowth has been studied extensively in *Xenopus*

embryo¹³. However, little is known about induction of the presumptive tail bud at the ventral margin of the gastrula. To approach this question we investigated the capacity of marginal cells to induce tail structures by transplanting portions of the blastula or gastrula margin into the animal pole; a territory located far from the region that the grafted cells originated from, and far from the zebrafish dorsal organizer (Fig. 2a). Whereas grafts of dorsal margin lead to the development of ectopic axial structures (notochord and floor plate, Fig. 2b, c; 78.6%, $n = 42$), grafts of ventral margin induce the formation of secondary tails (Fig. 2d–g and Table 1). These structures extend occasionally up to the trunk. Induced tails contain mesodermal and ectodermal derivatives, such as somites (Fig. 2d), blood cells, neural tube (Fig. 2f), and ventral and caudal fins (Fig. 2d). Axial structures (notochord, floor plate, ventral neural tube), the formation of which requires the activity of the dorsal organizer, are absent (Fig. 2d, g).

Secondary tails comprise cells from the donor, labelled with fluorescein-dextran, as well as unlabelled host cells (Fig. 2e). This

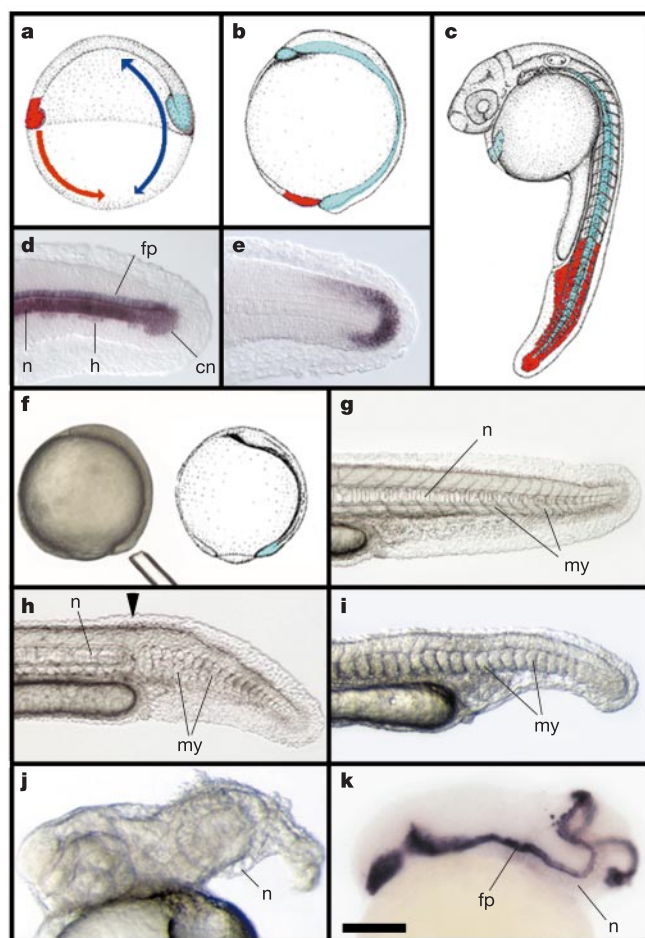


Figure 1 Contribution of axial and non-axial tissues to tail formation. **a, b**, Position and movement of ventral (red) and dorsal (blue) marginal cells at the shield stage (**a**), at the end of gastrulation (**b**) and at 24 h after fertilization (**c**). The dorsal margin contributes to axial structures (blue); ventral margin to non-axial tissues (red). **d, e**, Labelling of parts of the tail at 24 h after fertilization. Axial parts are labelled by *shh* and *CA2-IX* (**d**); non-axial by *eve* (**e**). **f–h**, Surgical extirpation with a needle removing the dorsal margin (**f**; blue) results in loss of axial structures posterior to the anus (arrowhead in **h**); compare the experimental embryo (**h**) with wild type (**g**). **i**, Tail of a ventralized embryo (10 pg *bmp4* RNA) lacking axial structures. **j**, Embryo injected with 0.6 ng *frzb* RNA. **k**, Axial territory labelled with *shh*. cn, chordo-neural hinge; fp, floor plate; h, hypochord; my, myotomes; n, notochord. Scale bar: 100 μ m (**d, e**); 150 μ m (**g–k**); 300 μ m (**f**).

shows that the graft has organizing properties as it induces neighbouring tissues of the host to form ectopic structures, including structures that it would otherwise not contribute to (for example, fin fold epidermis). In accordance with Spemann's definition of an embryonic organizer¹, these observations support the existence in

the zebrafish embryo of a tail organizer located at the ventral margin and which is responsible for the formation of non-axial tail tissues. By heterochronic and isochronic transplantations (Table 1) we show that this tail organizer forms as early as the sphere stage and is active up to late gastrula.

Secreted signalling molecules such as Wnt8 (ref. 14), BMPs (Bmp2b, Bmp4, Bmp7; involved in dorso-ventral axis formation⁹) and Nodal (Znr1/cyclops, Znr2/squint; needed for the induction of mesendoderm and the establishment of the anterior-posterior axis^{9,15}) display a strong overlap of their expression at the ventral margin of the zebrafish blastula (Supplementary Fig. S2). Moreover, loss of function of either one of these signalling pathways prevents development of the tail. Decrease of BMP signalling in heterozygous *Smad5/somitabun* mutant embryos¹⁶ or in weakly dorsalized mutants such as *tolloid/mini fin*¹⁷ results in adults devoid of a tail (Supplementary Fig. S3a). Strong inhibition of the BMP signalling pathway (BMP mutant embryos¹⁸; misexpression of a BMP inhibitor such as *noggin1* (ref. 19)) gives rise to embryos that develop axial structures but that are devoid of the non-axial part of the tail (Supplementary Fig. S3b, e–i). Similar phenotypes are observed after Wnt8 signalling pathway inactivation through morpholino knock down (Supplementary Fig. S3c), misexpression of a specific Wnt inhibitor (Fig. 1j, k; see also Supplementary Fig. S3j, k) or in embryos deficient for the Wnt8 locus¹⁰. Finally, the complete loss of the Nodal signalling pathway, through misexpression of a competitive inhibitor such as *antivin*¹⁵ or in embryos lacking both maternal and zygotic activity of the Nodal co-receptor *Oep*⁹, prevents both axis and tail formation (Supplementary Fig. S3d). Altogether these loss-of-function phenotypes suggest that BMP, Nodal and Wnt8 are potential candidates for the tail-organizing activity.

We investigated this hypothesis through misexpression of these factors either alone or in combination, by injection of sense RNA in an animal pole blastomere at the 128–256-cell stage. As described previously¹⁵, misexpression of *znr1* or *znr2* results in inducing ectopic axial structures in place of the anterior neur ectoderm (97.4%, $n = 39$). Similar observations were done with all factors stimulating the Activin/Nodal signalling pathway (data not shown). On the other hand, injection of up to 250 pg of sense RNA coding for Bmp4 in an animal pole blastomere does not induce any ectopic structures (Table 2). In contrast, injection of various combinations of *bmp4/znr1*, *bmp4/wnt8* or *bmp4/znr1/wnt8* RNAs at the animal pole resulted in the formation of secondary tails well patterned along their dorso-ventral and anterior-posterior axis (Fig. 2h–j and Table 2; see also Supplementary Fig. S4). The same phenotypes were generated by co-injection of other factors stimulating BMPs (Bmp2b, Bmp7, CA-BRIA²⁰) and Nodal (Znr2, Activin- β b, TaramA^p (ref. 21), CA-Smad2 (ref. 22)) signalling pathways.

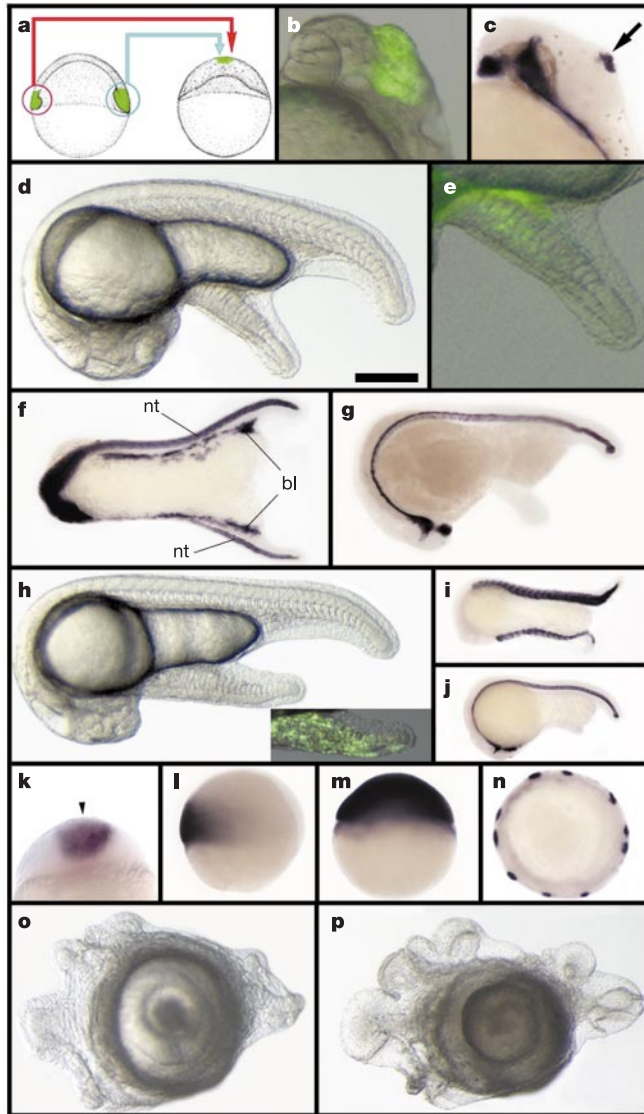


Figure 2 Identification of the tail organizer. **a–c**, Transplantation of dorsal margin (fluorescently labelled donor) to the animal pole of an unlabelled host (**a**; blue) induces ectopic axial structures (**b**) in the head at 30 h after fertilization (identified with *shh* in **c**; arrow). **d, e**, Transplantation of ventral margin (red in **a**) leads to formation of ectopic tail devoid of axial structures (**d**) comprising donor (fluorescent) and recruited (unlabelled) host cells (**e**). **f, g**, Induced tails contain blood (bl) and neural tube (nt) (**f**) but no axial structures (**g**) labelled with *shh*. **h**, Induction of ectopic tail after single-cell injection at the animal pole of a 128–256-cell-stage embryo with *bmp4/znr1/gfp* RNAs (200, 10 and 100 pg, respectively). Inset indicates that the tail comprises both GFP-labelled cells as well as unlabelled recruited cells. **i, j**, Induced tails contain muscles (**i**; labelled with *myoD*) but no axis (**j**; revealed with *shh*). **k**, Localized injection of *bmp4/znr1* (200 and 10 pg, respectively) RNAs induces ectopic ventral margin (labelled with *eve1*; arrowhead) at early blastula. **l, m**, General misexpression induces *eve1* expression in the whole blastula (**m**) (compare with wild type in **l**). **n**, Twenty-four hours after general misexpression of *bmp4/znr1*, *eve1* labelling identifies multiple tail buds. **o, p**, These tail buds differentiate after 36 h (**o**) into huge tails with multiple fin folds, myotomes but no axial structures, which at 48 h (**p**) fuse into a unique folded hypertrophic tail fin. Scale bar: 200 μ m (**b, c, e, o, p**); 250 μ m (**d, h**); 300 μ m (**d, h, k**); 350 μ m (**f, g, l–n**); 800 μ m (**i, j**).

Table 1 Tail-organizing activity of ventral marginal cells at blastula and gastrula stages

Stage of the donor	Stage of the host	Induction of ectopic tail (%)	
Sphere/dome (early blastula)	Sphere/dome	57.9	($n = 38$)
Shield (early gastrula)	Sphere/dome	66.1	($n = 59$)
75% epiboly (mid-gastrula)	Sphere/dome	48	($n = 27$)
100% epiboly (end of gastrula)	Sphere/dome	14.3	($n = 28$)
	Shield	20	($n = 60$)

Cells of donor embryos at various blastula and gastrula stages were grafted isochronically or heterochronically into host embryos at the sphere/dome or the shield stage. Inductions of ectopic tail are expressed in per cent; the number of grafts analysed is indicated in brackets. Inductive activity is maximal at the shield stage and decreases until the end of gastrulation. Heterochronic transplantation of ventral margin from an embryo at the onset of gastrulation to the animal pole of an early blastula embryo is more efficient than isochronic transplantation at the shield stage, probably because the animal pole is still a completely naive territory with uncommitted cells at the early blastula stage. We note that for isochronic transplantations at the shield stage, as previously described²⁸, embryos that do not develop tails form ectopic otic vesicles (35 out of 40), indicative of a posteriorization of the anterior neural plate. This probably results from grafts that do not contain the most ventral marginal cells (the no convergence no extension zone) but which are slightly more lateral.

Table 2 Comparison of tail-inducing abilities of *Znr1*, *Bmp4* and *Wnt8*

Ligands (pg)			Tail (%)	<i>eveI</i> (%)	<i>MyoD</i> (%)	Carbonic anhydrase (%)
<i>Bmp4</i>	<i>Znr1</i>	<i>Wnt8</i>				
–	15	–	0 (n = 39)	ND	ND	ND
10	5	–	49.9 (n = 115)	96.7 (n = 61)	100 (n = 34)	31 (n = 53)
10	15	–	29 (n = 127)	90.7 (n = 54)	100 (n = 40)	33.3 (n = 50)
10	45	–	18.5 (n = 148)	75.5 (n = 53)	100 (n = 38)	25 (n = 63)
50	5	–	53.6 (n = 144)	91.7 (n = 60)	100 (n = 48)	84.2 (n = 62)
50	15	–	48.3 (n = 147)	100 (n = 66)	100 (n = 49)	39.1 (n = 51)
50	45	–	47.1 (n = 82)	96.8 (n = 94)	100 (n = 34)	14.3 (n = 27)
–	–	100	0 (n = 113)	0 (n = 29)	ND	ND
50	–	100	53.9 (n = 76)	100 (n = 34)	93.5 (n = 31)	90 (n = 10)
250	–	–	0 (n = 43)	0 (n = 23)	ND	ND
250	5	–	51.6 (n = 70)	100 (n = 65)	100 (n = 14)	36.4 (n = 36)
250	15	–	58.8 (n = 145)	98.4 (n = 64)	100 (n = 53)	76.9 (n = 48)
250	45	–	54 (n = 145)	100 (n = 81)	100 (n = 38)	25 (n = 67)
250	–	100	56 (n = 89)	100 (n = 40)	95.2 (n = 21)	86.4 (n = 22)
250	15	100	42.4 (n = 144)	100 (n = 49)	100 (n = 16)	85.7 (n = 14)
–	–	–	*76.3 (n = 80)	–	–	–

Different combinations of ligands were injected at the animal pole of 128–256-cell-stage embryos. One-third of the injected embryos were fixed at mid-gastrula and stained with *eveI*. Tail induction was scored on morphological criteria on the remaining two-thirds of the embryos at 24 h after fertilization. Induction of *myoD* and carbonic anhydrase expression was scored in these induced tails. All numbers are percentages, with total number of embryos indicated in brackets. ND, not determined.

* Tail frequency observed in the case of the triple injection of *Znr1*, *Bmp4* and *Wnt8* after removal of morphologically uninterpretable (ball-shaped embryos) phenotypes.

By co-injection of green fluorescent protein (*gfp*) RNA we showed that induced secondary tails contain labelled cells corresponding to the progeny of the injected blastomere, as well as unlabelled cells, recruited among animal pole cells (Fig. 2h). Differentiated tissues in these ectopic tails were identified by morphological criteria and by *in situ* hybridization using tissue-specific markers. They contain cells of mesodermal and ectodermal origin, such as muscles (Fig. 2i), blood (Supplementary Fig. S4a), ventral and caudal fins (Supplementary Fig. S4b), and dorsal spinal cord neurons (Supplementary Fig. S4c). They express at their posterior tip the tail-bud marker *eveI*²³ (Supplementary Fig. S4d). These ectopic tails share the same characteristics as those generated after transplantation of ventral marginal cells at the animal pole (Fig. 2d–g). Furthermore, they are devoid of axial structures: notochord, floor plate (Fig. 2j) or ventral neurons (Supplementary Fig. S4c).

For different combinations of these ligands we compared their tail-inducing ability. In each case we scored the induction of a gastrula ventral margin marker (*eveI*), as well as the expression of markers for somitic muscles and blood at 24 h after fertilization. We first observed that *Bmp4* misexpression with *Nodal* and/or *Wnt8* is indispensable for ectopic tail formation. Secondly, for any concentration and any combination of ligands, *eveI* is massively induced (Table 2). This is also the case at 24 h after fertilization for induction of somitic muscles. Triple injection of *bmp4/znr1/wnt8* caused severe malformations in 44% of embryos (Table 2), precluding a morphological interpretation of the phenotype. However, out of the remaining embryos, most (76.3%, Table 2) displayed ectopic tails. This study shows that under proper stimulation of BMP and *Nodal*, BMP and *Wnt8*, or BMP, *Nodal* and *Wnt8* signalling pathways, cells of the animal pole share the same organizing properties as the ventral margin, and become fated to tail bud. Ectopic induction of ventral marginal cell fate occurs as early as the high stage, before the initiation of endogenous *eveI* expression at the ventral margin (Fig. 2k). Therefore, cells of the embryos are able to respond to tail-organizing signals (BMP, *Nodal* and *Wnt8*) immediately after the mid-blastula transition when the zygotic genome starts to be transcribed. This suggests that the induction of the tail organizer, which derives from the ventral margin, occurs as soon as the endogenous BMP, *Nodal* and *Wnt8* signalling pathways stimulate marginal cells. Finally, general misexpression of BMP and *Znr1* results in a massive induction of *eveI* in all cells of the blastula (Fig. 2l, m; 100%, 36 out of 36). Most of these embryos display strong early embryonic defects and lethality at the end of gastrulation. The few individuals surviving after 24 h (1%, 8 out of 812)

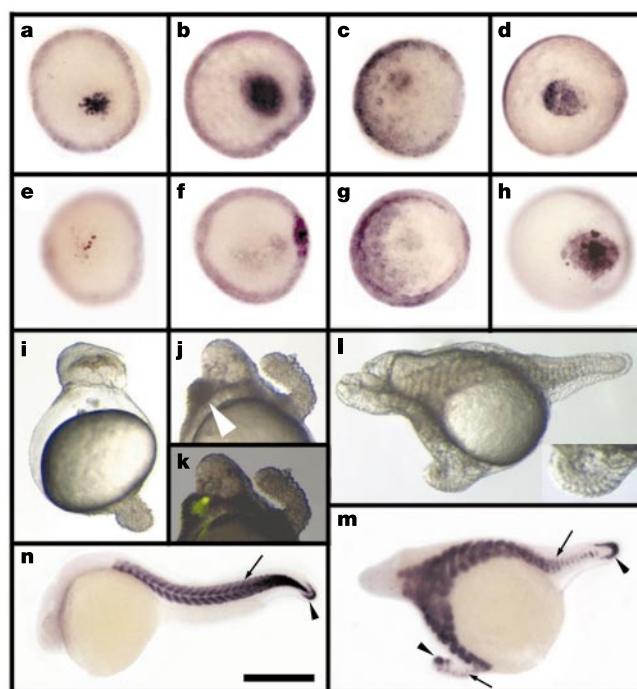


Figure 3 Molecular mechanisms underlying tail induction by BMP, *Nodal* and *Wnt8*. **a–d**, Single-cell injection in an animal pole blastomere of *znr2* RNA (1 pg) induces expression of *znr2* (**a**; 100%, 26 out of 26), *znr1* (**b**; 100%, 25 out of 25), *bmp2b* (**c**; 92.3%, 24 out of 26) and *wnt8* (**d**; 70%, 21 out of 30). **e–h**, Similar injection of *wnt8* RNA (100 pg) induces expression of *znr1* (**e**; 44%, 14 out of 32), *znr2* (**f**; 48%, 24 out of 50), *bmp2b* (**g**; 89%, 23 out of 26) and *wnt8* (**h**; 90%, 18 out of 20). **i**, Complete inhibition of *Nodal* signalling by injection of *antiviral* RNA (0.6 ng) at the one-cell stage. **j**, Embryo treated as in **i** and further injected in an animal pole blastomere at the 128-cell stage with *bmp4/wnt8/gfp* RNAs (200, 100 and 100 pg, respectively). **k**, GFP-labelled cells, deriving from the injected clone, are located close to the head in a mass of undifferentiated cells (arrowhead in **j**). **l**, Embryo devoid of the dorsal organizer through injection of *bmp4* RNA (10 pg), further injected in an animal pole blastomere at the 128-cell stage with *bmp4/znr1* RNAs (200 and 10 pg, respectively) develops an ectopic tail showing differentiated myotomes (inset). **m**, Same embryo as **l** probed with *myoD* (arrow) and *eveI* (arrowhead); compare with wild type in **n**. Scale bar: 250 μm (**n**); 350 μm (**i–m**); 400 μm (**a–h**).

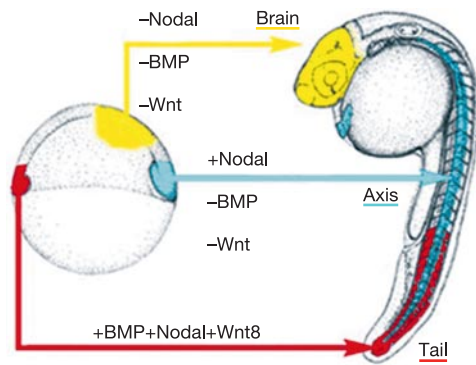


Figure 4 The zebrafish embryo is patterned by the combined activity of BMP, Nodal and Wnt signalling pathways. The formation of the head results from the triple inhibition of BMP, Nodal and Wnt. Formation of the axis (prechordal plate, chorda-mesoderm and ventral neural tube) results from the inhibition of BMP and Wnt and stimulation by Nodal. The formation of the tail depends on the triple stimulation of BMP, Nodal and Wnt8 signalling pathways.

consist of a ring of cells with multiple tail buds (as identified by labelling with *evel*; Fig. 2n), which further develop into multiple tails (Fig. 2o, p). These embryos differentiate myotomes as well as a huge, folded caudal fin. They lack all organs except for the tail and appear therefore nearly completely posteriorized.

Because BMP and Nodal and BMP and Wnt8 have comparable tail-inducing properties, we analysed their potential cross-regulation. Stimulation of the BMP signalling pathway in an animal pole blastomere has no effect on Nodal or Wnt8 expression (data not shown). However, misexpression of Nodal (*Znr1* or *Znr2*) results in strong induction of *znr1* and *znr2* (as previously shown²⁴) as well as *bmp2b* and *wnt8* expression (Fig. 3a–d). Similarly, injection of *wnt8* RNA induces expression of *znr1*, *znr2*, *bmp2b* and *wnt8*, albeit less efficiently than Nodal (Fig. 3e–h). To summarize, expression of Nodal at early developmental stages induces expression of *wnt8* and vice versa, in a positive auto-regulatory loop. As a consequence, injection of BMP (which is not able to induce a tail by itself but is absolutely required for its induction) with either Nodal or Wnt8 results in the concomitant stimulation of the three signalling pathways, leading to tail development. In accordance with this observation, inhibition of any of these signalling pathways prevents the formation of ectopic tails. As exemplified here in embryos overexpressing *antivin1*, a competitive inhibitor of the Nodal signalling pathway¹⁵, injection of a combination of BMP and Wnt8 RNAs is unable to induce formation of ectopic tails (Fig. 3i–k). Similarly, loss of Wnt8 activity through morpholino knock down prevents cells injected with BMP and Nodal from developing an ectopic tail (not shown). Nevertheless, although Nodal is able to induce both Wnt8 and *Bmp2b*, it is not able to induce tail by itself when injected in an animal pole blastomere. This is probably due to the fact that Nodal also induces strong expression of BMP inhibitors such as *chordin* and *noggin*.

Our previous observations (Fig. 1) suggested that the tail organizer acts independently of the dorsal organizer. To get direct evidence of this, we generated embryos devoid of a dorsal organizer through ventralization induced by BMP overexpression. As expected, misexpression of a combination of BMP and Nodal in an animal pole blastomere of these ventralized embryos results in the induction of a secondary tail (Fig. 3l–n). Similar observations were done after grafting of ventral margin at the animal pole of these ventralized embryos (data not shown).

Finally, structures induced after grafts of ventral most marginal cells or strong stimulation of BMP, Nodal and Wnt8 signalling pathways always comprise tail tissues, but they can occasionally

extend anteriorly up to the trunk. This suggests that, although the highest stimulation of BMP, Nodal and Wnt8 signalling pathways induces ventral most cell fates (giving rise to the most posterior territory) and is able to convert the whole embryo in a hypertrophic tail (Fig. 2o, p), weaker stimulation of BMP, Nodal and Wnt8 can gradually induce more lateral cell fates. This may explain the presence of trunk in the secondary induced structures.

In vertebrates, formation of the caudal part of the embryo suggests a tail-organizing role of the late Spemann organizer as it was described in different model organisms^{13,25–27}. We establish here (Fig. 4) that the development of the caudal part of the zebrafish embryo does not result from the activity of the dorsal organizer but from the activity of an independent organizer, the tail organizer, located at the ventral blastula margin and which results from the triple stimulation of BMP, Nodal and Wnt8 signalling pathways. □

Methods

Single-cell injections

All injected RNAs have been transcribed from a pCS2 + plasmid digested with *NotI* and synthesized with the SP6 RNA polymerase kit (Ambion). Each combination of ligands has been injected at the animal pole blastomere of a 128–256-cell-stage embryo together with *gfp* RNA (100 pg) in an agarose mould. Embryos were cultured at 28.5 °C in Danieau × 0.3 medium supplemented with 1% penicillin/streptomycin (Gibco, 15140-122).

Constructions

CA-BRIA is a constitutively active form of the BMP receptor²⁰. TaramA^P refers to a constitutively active form of Alk4/activin receptor type 1 (ref. 21). CA-Smad2 is constitutively active form of the activin/Nodal cytoplasmic signal transducer²².

Morpholino design

Antisense morpholino oligonucleotide (GeneTools, LLC) designed for zebrafish *Wnt8a* (5'-ACGCAAAAATCTGGCAAGGGTTTCAT-3') was injected into one- to four-cell-stage embryos. Morpholino was diluted in × 1 Danieau medium.

Fish strains

The wild-type strain used is a cross between *AB and Tübingen; embryos lacking both maternal and zygotic activity of *Oep* are from a cross of males and females homozygous for *oep*^{ts257} and rescued by injection of *oep* RNA at early embryonic stages. *mini fin* mutants are homozygous for the *ty130a* allele¹⁷.

Whole-mount *in situ* hybridization

All whole-mount *in situ* hybridizations were performed as described (see http://zfinfo.org/zf_info/zfbook/chapt9/9.82.html). The following markers were used to probe the tail at 24 h after fertilization. *shh* stains floor plate, notochord and chordo-neural hinge (Fig. 1d, k; see also Supplementary Figs S1a–d, S2c, g, j and S3b, c); collagen alpha 2 type IX (CA2-IX) stains notochord, floor plate and hypochord (Fig. 1d); *evel* (ref. 23) stains ventral marginal cells during gastrula and tail-bud cells at 24 h after fertilization (Figs 1e, 2k–n and 3m, n; see also Supplementary Fig. S4d); *islet1* stains Rohon–Beard neurons and motor neurons of the spinal cord (Supplementary Figs S1a–d and S4c); *sox19* stains the central nervous system (our own unpublished observations; see also Fig. 2f); *carbonic anhydrase* stains blood and otic vesicles (Fig. 2f and Table 2; see also Supplementary Fig. S4a); *myoD* stains somites (Figs 2i, 3m, n and Table 2); chondroitin sulphate proteoglycan protein 2 stains axial fin fold, heart and lens primordium (Supplementary Fig. S4b). (For information on the above zebrafish molecular genes see <http://zfinfo.org/cgi-bin/webdriver?Mval=aa-geneselect.app>.)

Surgical extirpation and transplantation

Surgical extirpation and transplantation experiments were performed by suction as described³. Donor embryos were injected at the 1–2-cell stage with fluorescein-dextran 10,000 MW (Molecular Probe D7137). Cells were transplanted from the margin of the donor embryo to the animal pole of the host embryo in × 1 Danieau medium supplemented with 2% penicillin/streptomycin. Transplantation needles (pulled from borosilicate glass capillaries; Harvard Apparatus GC120F-15) were cut to an approximate diameter of 80 µm. The number of grafted cells was between approximately 200 and 300. Embryos were cultured in small Petri dishes in Danieau × 0.3 medium supplemented with 2% penicillin/streptomycin at 28.5 °C.

Determination of the origin of transplanted blastula cells

Donor embryos were injected at the 1–2-cell stage with fluorescein-dextran 10,000 MW (Molecular Probe D7137) in one blastomere and rhodamine-dextran 40,000 MW (Molecular Probe D1840) in the other blastomere. Grafts have been performed as described above. Each of the donor–host coupled embryos were grown in a single dish. At the shield stage the dorso-ventral axis is morphologically recognizable. At this stage when donor embryos display fluorescein labelling on the ventral side and rhodamine labelling on the dorsal side, host embryos were analysed for the fluorescence of the graft (Supplementary Fig. S5). Green clones indicate a ventral origin of the graft at blastula stage

whereas red clones indicate a dorsal origin. Clones of mixed colour have been discarded because of their ambiguous dorso-ventral origin.

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WAVE2 is required for directed cell migration and cardiovascular development

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WAVE2, a protein related to Wiskott–Aldrich syndrome protein, is crucial for Rac-induced membrane ruffling, which is important in cell motility^{1–4}. Cell movement is essential for morphogenesis, but it is unclear how cell movement is regulated or related to morphogenesis. Here we show the physiological functions of WAVE2 by disruption of the *WAVE2* gene in mice. WAVE2 was expressed predominantly in vascular endothelial cells during embryogenesis. *WAVE2*^{−/−} embryos showed haemorrhages and died at about embryonic day 10. Deficiency in WAVE2 had no significant effect on vasculogenesis, but it decreased sprouting and branching of endothelial cells from existing vessels during angiogenesis. In *WAVE2*^{−/−} endothelial cells, cell polarity formed in response to vascular endothelial growth factor, but the formation of lamellipodia at leading edges and capillaries was severely impaired. These findings indicate that WAVE2-regulated actin reorganization might be required for proper cell movement and that a lack of functional WAVE2 impairs angiogenesis *in vivo*.

When a cell moves, the actin cytoskeleton is reorganized, and protrusive membrane structures such as lamellipodia and filopodia are formed at the leading edge⁵. These structures generate the locomotive force in migrating cells. Production of phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃) by the activation of phosphatidylinositol-3-OH kinase in response to chemoattractants is reported to be required for the formation of cell polarity and a leading edge^{6,7}. Accumulation of PtdIns(3,4,5)P₃ stimulates Rho-family small GTPases such as Rac and Cdc42, leading to the formation of a leading edge. Rho-family small GTPases are thought to regulate actin reorganization⁸. Rac and Cdc42 regulate the formation of lamellipodia and filopodia, respectively. Wiskott–Aldrich syndrome protein (WASP)-family proteins are key regulators that link extracellular stimuli to actin reorganization⁹.

To investigate the role of WAVE2 in cell migration and mouse development, we generated *WAVE2*-deficient mice by homologous recombination (Fig. 1a; Supplementary Fig. S1). Mice heterozygous for the *WAVE2* mutation were healthy and fertile. By embryonic day 10 (E10), mendelian ratios of wild-type, heterozygous and homozygous embryos were detected; however, there was a drastic decrease in the number of homozygotes at E10.25–E10.5, and no live *WAVE2*^{−/−} embryos were found at E11.5 (data not shown). These *WAVE2*^{−/−} embryos did not express WAVE2 protein (Supplementary Fig. S1c), indicating that these embryos carry a null mutation. At E8.5 there was no significant difference in appearance between wild-type and *WAVE2*^{−/−} embryos; however, as development proceeded, *WAVE2*^{−/−} embryos showed developmental delay and growth retardation (compare Fig. 1b with c). Wild-type and *WAVE2*^{−/−} embryos contained a similar number of somites at E8.5

(data not shown), but at E9.5, *WAVE2*^{-/-} embryos had fewer somites than did wild-type embryos (21.1 ± 3.4 in wild type; 16.7 ± 3.3 in *WAVE2*^{-/-}; $P < 0.05$). These *WAVE2*^{-/-} embryos never had more than 29 somites, whereas wild-type littermates had more than 30 somites. At E10.5, *WAVE2*^{-/-} embryos were very fragile and occasionally had oedema of the head (5% of *WAVE2*^{-/-} embryos, $n = 39$), and the yolk sacs of *WAVE2*^{-/-} embryos appeared pale with few blood cells (data not shown). The hearts of *WAVE2*^{-/-} embryos were small and incompletely looped compared with their wild-type littermates (compare Fig. 1d with e), and dilation of the pericardial cavities was apparent (Fig. 1e, black arrowhead). In many cases (57% of *WAVE2*^{-/-} embryos, $n = 28$), the *WAVE2*^{-/-} embryos had haemorrhages in the head regions, pericardial cavities, hearts and ventral parts of the trunk (Fig. 1e, white arrowheads). *WAVE2*^{-/-} embryos died at about E10, possibly because of haemorrhage or oedema of the heart, indicating that *WAVE2* is required for normal embryonic development.

During early development, *WAVE2* expression was detected preferentially in dorsal aortas and ingrowths into the neural tube (Fig. 1f) and was coincident with the expression of platelet-endothelial cell adhesion molecule (PECAM), which was used as an endothelial marker (Fig. 1g, h), but not α -smooth muscle actin (data not shown). Similarly, *WAVE2* was produced in endocardial cells but not in the myocardial layer (Fig. 1i–k). No signal was detected in identical experiments with *WAVE2*^{-/-} embryos (data not shown). In contrast, *WAVE1* was enriched in neural tubes but not in vascular endothelial cells (Fig. 1l–n). *WAVE3* was not detected during this developmental stage (data not shown). *WAVE2* was the only *WAVE* family protein that was preferentially expressed in endothelial cells during the early stages of development.

During embryogenesis, the cardiovascular system develops through a series of complex events¹⁰. The development of embryonic vasculature takes place through two distinct processes: vasculogenesis and angiogenesis. During vasculogenesis, the primitive vascular plexus was formed almost normally in *WAVE2*^{-/-} embryos with 18 somites (Fig. 2a–d; Supplementary Fig. S2a–e). The intersomitic vessels of *WAVE2*^{-/-} embryos were also formed, although they were slightly disorganized in comparison with those of wild-type embryos (compare Fig. 2c with d, white arrowheads; Supplementary Fig. S2f).

During angiogenesis, in the yolk sacs of *WAVE2*^{-/-} embryos with 22 somites, the primary capillary plexus was remodelled and the larger vessels—the vitelline arteries and veins—were formed (Fig. 2e, f, large arrowhead; Supplementary Fig. S2b), but other capillaries were much smaller than those of wild-type embryos and were occasionally discontinuous (Fig. 2f, small arrowheads; Supplementary Fig. S2b). In the heads of *WAVE2*^{-/-} embryos with 22 somites, the larger vessels did not develop appropriately (compare Fig. 2g with h, large and small arrowheads; Supplementary Fig. S2a), and the head capillaries were less dense and branched in *WAVE2*^{-/-} embryos than in wild-type embryos with the same number of somites (Supplementary Fig. S2d). In addition, the capillary network was occasionally disrupted (Fig. 2h, arrows). The major vessels of the trunk region, such as the dorsal aorta and anterior cardinal vein, were relatively small in *WAVE2*^{-/-} embryos (compare Fig. 2i with j, black arrowhead and arrow; Supplementary Fig. S2c). In addition, in some *WAVE2*^{-/-} embryos (33.3% of *WAVE2*^{-/-} embryos, $n = 12$) with 22 somites, the intersomitic vessels seemed disorganized (compare Fig. 2i with j, white arrowheads). Moreover, angiogenic sprouting was inhibited at the most dorsal part of the somites and the neural tube of *WAVE2*^{-/-} embryos and in the labyrinthine region of *WAVE2*^{-/-} placentas (Fig. 2i–p; Supplementary Fig. S2g). Thus, angiogenic remodelling and sprouting did not take place correctly in *WAVE2*^{-/-} embryos, yolk sacs and placentas. In contrast, smooth muscle cells were recruited normally in *WAVE2*^{-/-} embryos in comparison with wild-type embryos with

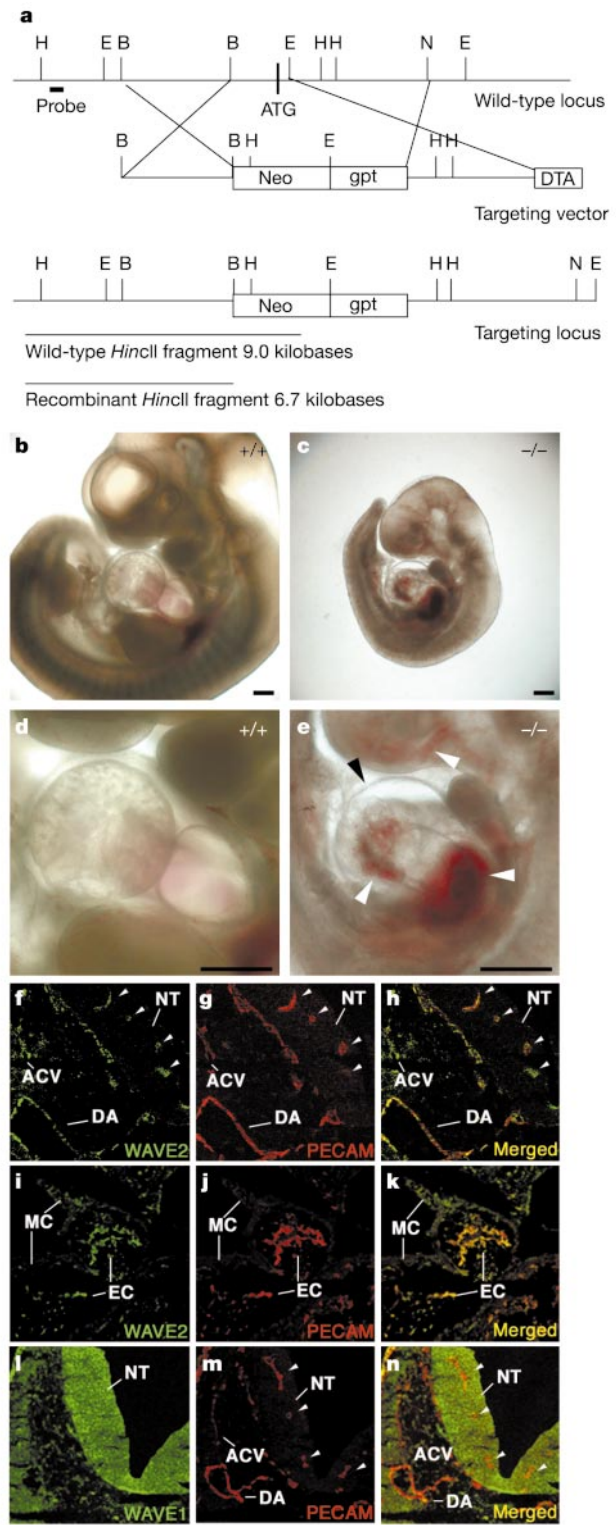


Figure 1 Targeted disruption of the *WAVE2* gene. **a**, Restriction map. gpt, xanthine/guanine phosphoribosyl transferase gene. **b–e**, Whole-mount views of E10.5 embryos. **d**, **e**, Higher magnifications of **b** and **c**, respectively. White and black arrowheads in **e** indicate haemorrhages and dilation of the pericardial cavity, respectively. **f–n**, Transverse sections at the level of the dorsal aorta (**f–h** and **l–n**) and the heart (**i–k**) in wild-type embryos with 27 somites were double-labelled for PECAM and *WAVE2* (**f–k**) or *WAVE1* (**l–n**). Arrowheads indicate endothelial cells sprouting into neuroepithelium. ACV, anterior cardinal vein; DA, dorsal aorta; EC, endocardium; MC, myocardium; NT, neural tube. Scale bars, 300 μ m.

the same number of somites (compare Fig. 2q with r). In addition, similarly to vascular development, endothelial lineage in the heart showed abnormal development, but myocardial walls were relatively normal in *WAVE2*^{-/-} embryos (Supplementary Fig. S3).

Next we examined the angiogenic properties of *WAVE2*^{-/-} embryos in a para-aortic splanchnopleural (P-Sp) culture system^{11,12}. In this culture system, PECAM-positive cells from explants of E9.5 embryos formed a sheet-like structure (vascular bed) and subsequently a network structure (vascular network) (Fig. 2s). Vasculogenesis in the P-Sp culture system has been defined as the process of formation of a vascular bed, and angiogenesis as the subsequent process that forms a vascular network, because Tie2 expression is low in endothelial cells of a vascular bed and high in those of a vascular network^{11,12}. In P-Sp cultures from *WAVE2*^{-/-} embryos, vascular beds, but not vascular networks, were formed (compare Fig. 2s with t; Supplementary Fig. S2h). Our collective results indicate that the *WAVE2*-null mutation impairs angiogenesis even in this *in vitro* culture system. Impaired vessel formation in *WAVE2*^{-/-} embryos would therefore be the primary effect of disruption of *WAVE2* in endothelial cells.

To understand the cellular basis of the endothelial defects in *WAVE2*^{-/-} embryos, we used electron microscopy to examine the

mutant embryonic vessels and hearts. The endothelial cells in the aorta and head capillaries of *WAVE2*^{-/-} embryos were flatter than cells from wild-type embryos (compare Fig. 3a, c with b, d, respectively; Supplementary Fig. S2j). Surprisingly, *WAVE2*^{-/-} embryos showed some gaps in the vascular endothelial cells of head capillaries and in endothelium of the heart (compare Fig. 3c, e, g, i with d, f, h, j, respectively; Supplementary Fig. S2k, l). Scanning photomicrographs clearly confirmed these defects in endothelial cells, and leaking erythrocytes were visible (Fig. 3f). However, the structures of cell-cell junctions between neighbouring endothelial cells (Fig. 3a–d, arrowheads), the recruitment of peri-endothelial cells and the interaction between endothelial cells and their surrounding cells were normal in appearance (Fig. 3a, b, arrows), indicating that these gaps might be formed before the formation of junctions. In addition, fewer membrane protrusions were present in the head capillary vessels of *WAVE2*^{-/-} embryos than in those of wild-type embryos (compare Fig. 3c, e with d, f, respectively). Collectively, electron microscopic analysis revealed several defects in the morphology of endothelial cells in *WAVE2*^{-/-} embryos, the presence of which probably explains the haemorrhages and oedema observed in the head and heart.

To examine whether defective cardiovascular development in

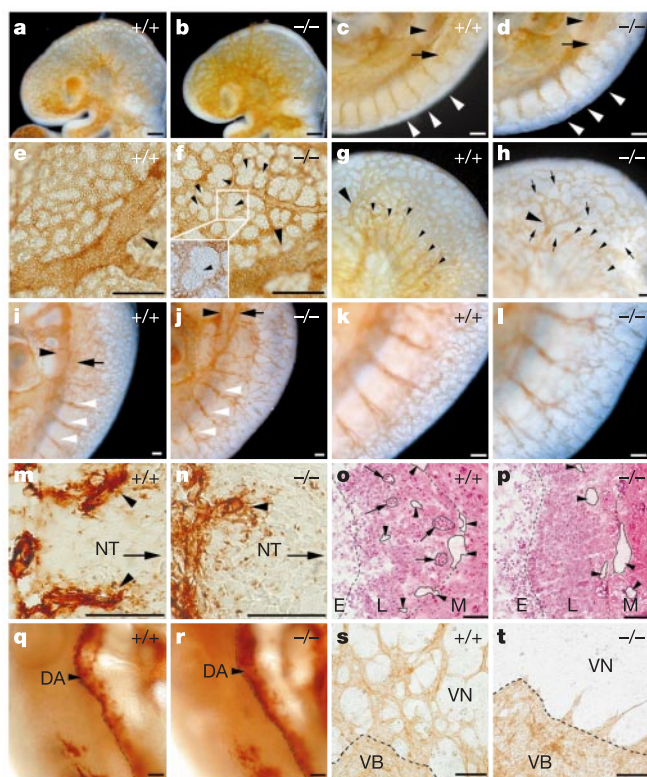


Figure 2 Defects in vascular remodelling in *WAVE2*^{-/-} embryos. **a–l**, Whole-mount PECAM immunostaining. **a–d**, Eighteen somites; **e–l**, 22 somites. **a, b, g, h**, Head region. Large arrowheads indicate internal carotid artery, and small arrowheads branches of the anterior cardinal veins. Arrows in **h** indicate discontinuous networks. **e, f**, Yolk sacs. Arrowheads, vitelline artery (large) or discontinuous capillaries (small in **f**). **c, d, i–l**, Trunk region. **k, l**, Magnification of **i** and **j**, respectively. Arrows, anterior cardinal vein; arrowheads, dorsal aorta (black) or intersomitic vessels (white). **m, n**, PECAM-stained transverse sections through the head region at the 27-somite stage. Arrow, direction of sprouting; arrowheads, sprouting capillaries. **o, p**, Haematoxylin–eosin-stained sections of placentas. Arrows, embryonic vessels (defined by the presence of nucleated cells); arrowheads, maternal vessels. **q, r**, Whole-mount immunostaining of α -smooth muscle actin at the 26-somite stage. **s, t**, Whole-mount PECAM immunostaining of P-Sp cultures. DA, dorsal aorta; E, embryonic tissue; L, labyrinthine region; M, maternal tissue; NT, neural tube; VB, vascular bed; VN, vascular network. Scale bars, 100 μ m.

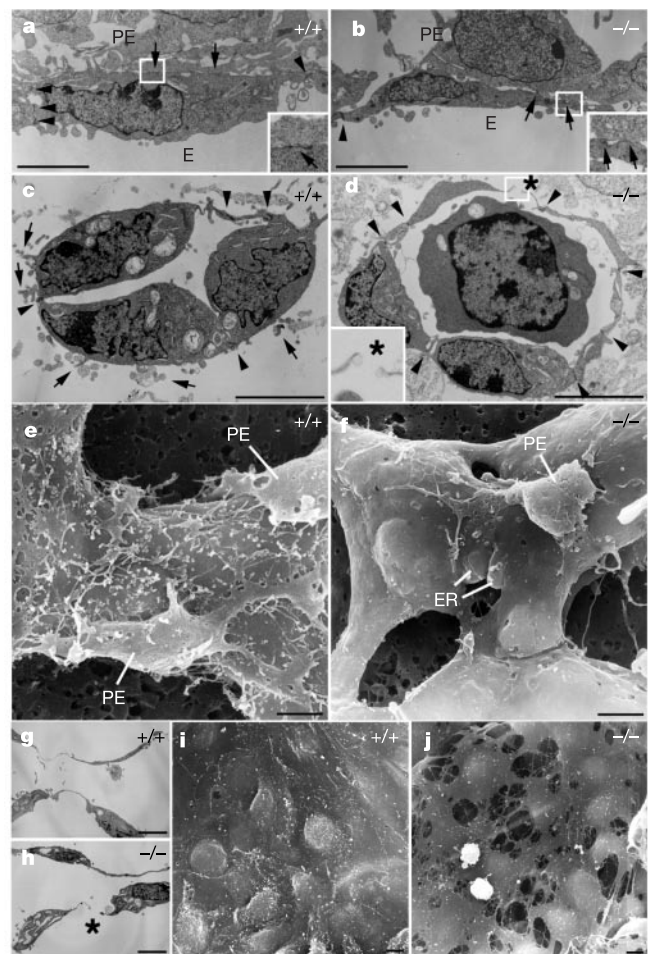


Figure 3 Electron-microscopic analysis. Transmission scanning electron photomicrographs (**a–d, g, h**) and scanning electron photomicrographs (**e, f, i, j**) of endothelial cells. **a, b**, Dorsal aorta in the trunk region. **c–f**, Head capillary. **g–j**, Endocardium in the heart. Arrows in **a** and **b** indicate junctions between the endothelial cell and the peri-endothelial cell; arrows in **c** show membrane protrusions. Arrowheads, junctions between neighbouring endothelial cells; asterisk, gap; E, endothelial cell; ER, erythrocyte; PE, peri-endothelial cell. Scale bars, 5 μ m.

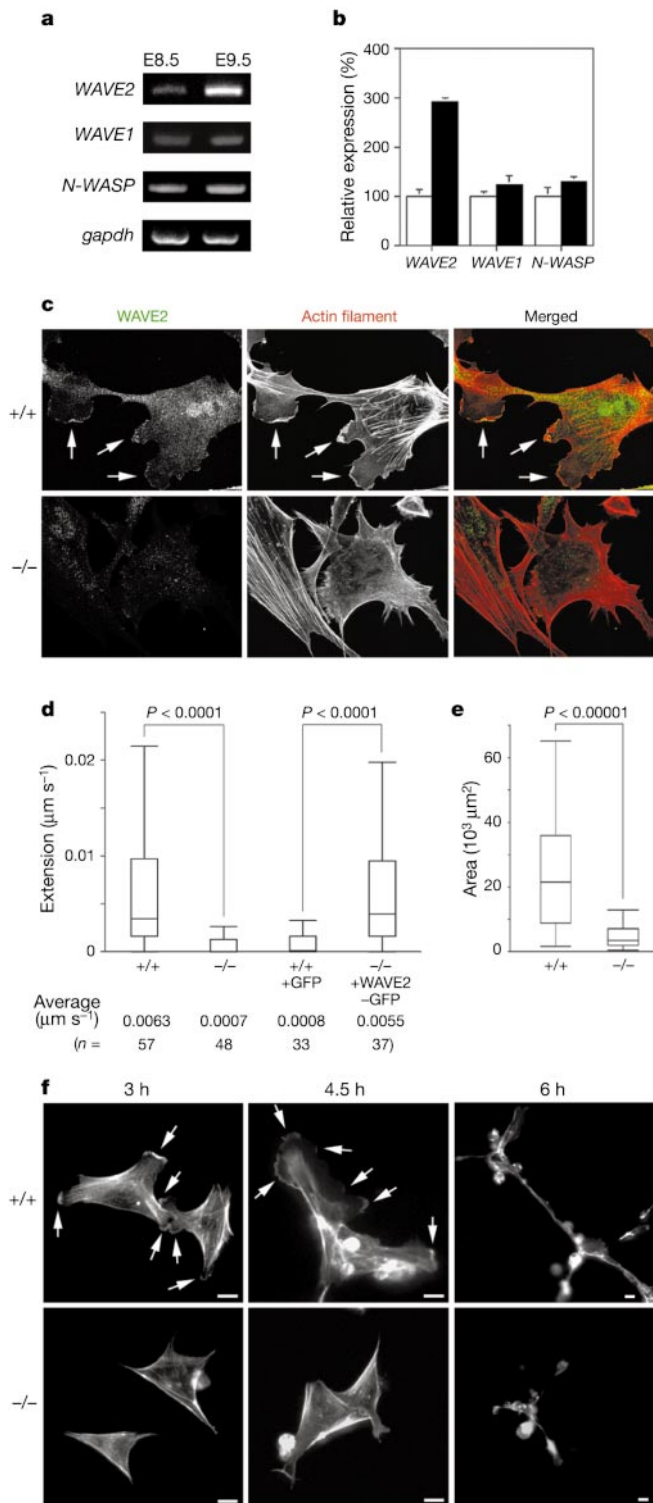


Figure 4 Physiological functions of WAVE2 in endothelial cells. **a**, Analysis of gene expression of E8.5 and E9.5 endothelial cells by RT-PCR. *gapdh*, gene encoding glyceraldehyde-3-phosphate dehydrogenase. **b**, Relative intensity levels of PCR products were measured and normalized for GAPDH levels. Open bars, E8.5 cells; filled bars, E9.5 cells. **c**, Endothelial cells from wild-type and *WAVE2*^{-/-} embryos at E9.5 were treated with VEGF (10 ng ml⁻¹) for 10 min. Arrows indicate peripheral membrane ruffles. **d**, Extension speed of the edge of the cell membrane towards VEGF. **e**, **f**, Capillary formation on Matrigel under VEGF. **e**, Plot of capillary area. **f**, Endothelial cells were cultured on Matrigel and stained with phalloidin. Arrows indicate lamellipodial protrusions. Scale bars, 30 μm .

WAVE2^{-/-} embryos was due to the impaired migration of endothelial cells, we isolated endothelial cells from E9.5 embryos by cell sorting with anti-PECAM antibody. We confirmed that endothelial cells were properly isolated from both wild-type and *WAVE2*^{-/-} embryos by immunofluorescent analysis with anti-PECAM and anti-Tie2 antibodies (data not shown) and polymerase chain reaction with reverse transcription (Supplementary Fig. S4a). In wild-type endothelial cells, *WAVE2* expression was higher than in control cells (Supplementary Fig. S4a), and was markedly higher at E9.5 than at E8.5 (Fig. 4a, b). In contrast, the expression of *WAVE1* and *N-WASP*, the genes encoding the other WASP-family proteins involved in tubulogenesis¹³, was slightly greater at E9.5 than at E8.5.

We further examined actin reorganization in isolated endothelial cells by treatment with vascular endothelial growth factor (VEGF) for 10 min. About 85% of wild-type endothelial cells responded to VEGF with peripheral membrane ruffles, but few (less than 10%) *WAVE2*^{-/-} endothelial cells had peripheral membrane ruffles as judged by staining of actin filaments (Fig. 4c; Supplementary Fig. S4b). At the edge of lamellipodial structures, accumulation of *WAVE2* was observed in immunofluorescent analyses (Fig. 4c). These results indicate that *WAVE2* might be required for VEGF-induced actin filament reorganization in endothelial cells.

We next examined the response of wild-type and *WAVE2*^{-/-} endothelial cells to VEGF by time-lapse microscopy. VEGF was applied to cells by micropipette to produce a gradient of concentrations of VEGF; most endothelial cells isolated from both wild-type and *WAVE2*^{-/-} embryos responded to VEGF (Supplementary Fig. S5a–c, movies in Fig. S6a and S6b). About 90% of wild-type endothelial cells responded to VEGF by extending lamellipodia (Supplementary Fig. S5a, c; movie in Supplementary Fig. S6a). In contrast, about 90% of *WAVE2*^{-/-} endothelial cells also responded to VEGF by producing tiny peripheral membrane ruffles (Supplementary Fig. S5b, c; movie in Supplementary Fig. S6b). The tiny ruffles responding to VEGF in *WAVE2*^{-/-} endothelial cells did not persist and soon disappeared, explaining the decrease in lamellipodial structure formation after treatment with VEGF for 10 min (Fig. 4c). When we measured the extension speed of the edge of the cell membrane, the speed of cell membrane extension responding to VEGF was greatly reduced in *WAVE2*^{-/-} endothelial cells (0.0007 $\mu\text{m s}^{-1}$) in comparison with the speed of wild-type endothelial cells (0.0063 $\mu\text{m s}^{-1}$) (Fig. 4d). Neither type of endothelial cell responded to control buffer (data not shown). The defect in membrane extension observed in *WAVE2*^{-/-} endothelial cells was restored by ectopic expression of *WAVE2* fused with green fluorescent protein in a manner similar to that of wild-type endothelial cells (Fig. 4d; Supplementary Fig. S5d, movies in Supplementary Fig. S6c–f). Thus, signals upstream of *WAVE2* seem to be normal in *WAVE2*^{-/-} endothelial cells. Indeed, both wild-type and *WAVE2*^{-/-} endothelial cells were able to induce cell polarity in response to VEGF, which were confirmed by PtdIns(3,4,5)P₃ production at the side facing VEGF (Supplementary Fig. S7). Collectively, *WAVE2*^{-/-} endothelial cells have all the signalling components, except *WAVE2*, necessary for lamellipodial protrusion.

We examined whether *WAVE2*^{-/-} endothelial cells showed morphological changes in capillary formation on Matrigel¹⁴. In this assay, endothelial cells differentiate into vascular structures; those are necessary for sprouting and tube formation *in vivo*. Wild-type endothelial cells formed capillary-like structures, whereas *WAVE2*^{-/-} endothelial cells did not form capillary structures as judged by the area of the cells (Fig. 4e, f). When cultured on Matrigel, wild-type endothelial cells formed lamellipodial protrusions (Fig. 4f, arrows), and then migrated along neighbouring cells to form capillary structures. In contrast, lamellipodia were scarcely observed, and capillary formation was severely inhibited in *WAVE2*^{-/-} endothelial cells. Thus, *WAVE2* would regulate endothelial cell migration through the reorganization of actin during embryogenesis, resulting in the formation of a complex vascular

plexus. We also confirmed that actin reorganization induced by WAVEs is required for the migration of human umbilical vein endothelial cells (Supplementary Fig. S8).

Here we have shown that WAVE2 is essential for cardiovascular development during embryogenesis. However, we do not know the physiological function of WAVE2 in other tissues or organs. Production of a conditional knockout of WAVE2 will be helpful in solving these issues. □

Methods

Targeted disruption of the WAVE2 gene

A mouse 129 Sv/J genomic library was screened for WAVE2, and several fragments containing the exon including ATG were isolated. In our targeting vector (Fig. 1a), a 5.2-kilobase DNA fragment encompassing the start codon of the WAVE2-coding sequence was deleted and replaced with a neomycin-resistance minigene. The linearized targeting vector was electroporated into 129/Ola embryonic stem cells. Two independent heterozygous embryonic stem cell clones were used to generate chimaeric mice by blastocyst injection, and mutant animals were bred on a mixed 129/Ola × C57BL/6J background. Genotyping was done by Southern blotting (Supplementary Fig. S1a) and PCR assays (Supplementary Fig. S1b). For Southern blot analysis, genomic DNA was digested with *HincII* and hybridized with a 5' probe. The 5' probe corresponded to a 0.5-kilobase *PstI*-*EcoRV* DNA fragment isolated from the WAVE2 genomic clone. Primers for PCR analysis were 5'-TATGGCCTGTCCACTGTGAC-3', 5'-TCAGATCGCAGCCC TTGCTT-3' and 5'-CCTGTGCTCTAGTAGCTTACG-3'.

Antibodies

Polyclonal anti-WAVE1, anti-WAVE2 and anti-WAVE3 antibodies were prepared in rabbits immunized with the basic regions of human WAVEs (WAVE1, amino acids 180–246; WAVE2, amino acids 180–241; WAVE3, amino acids 181–246) expressed in *Escherichia coli*. Monoclonal anti-PECAM-1 antibody (clone MEC 13.3) was from Pharmingen and antibody against anti- α -smooth muscle actin (clone 1A4) was from Sigma. Polyclonal anti-Tie2 antibody was from Santa Cruz Biotechnology.

Immunohistochemical staining

Whole-mount immunohistochemistry was performed as described¹⁵. The sections were stained with the tyramide signal amplification biotin system (NEN Life Science Products).

Electron microscopy

Mouse embryos were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3. The head, heart and a small piece of body distal to the upper limb bud were removed from each embryo. Specimens were postfixed with 2% osmium tetroxide for 2 h, block-stained with 3% uranyl acetate for 2 h and embedded in epoxy resin. Thin sections were cut, stained with uranyl acetate and lead citrate and examined with a Hitachi H-800 transmission electron microscope. Observation with a scanning electron microscope was performed as described previously¹⁶.

Isolation of endothelial cell from E9.5 embryos

Each E9.5 embryo was dissected and incubated separately for 30 min with 0.1% collagenase (Sigma) in DMEM medium supplemented with 10% FCS at 37 °C. Cells were dispersed by pipetting and incubated for 5 min with 400 ng ml⁻¹ fluorescein isothiocyanate (FITC)-conjugated anti-CD31/PECAM (MEC13.3) antibody (Pharmingen) at 25 °C. Cells were then washed with PBS supplemented with 0.5 mM EDTA and 0.5% BSA. Cells were resuspended with 100 μ l PBS supplemented with 0.5 mM EDTA and 0.5% BSA with 10 μ l anti-FITC microbeads (magnetic beads) (Miltenyi Biotec GmbH) and incubated for 15 min on ice. These cells were applied to MACS MS separation columns on magnetic stands and washed with 1.5 ml PBS supplemented with 0.5 mM EDTA and 0.5% BSA. The cells bound to the magnetic column were collected by washing the column away from the magnetic stands. Cells were cultured from one embryo in one well of a collagen-coated 96-well plate with EGM-2 medium and the EGM-2 bullet kit (Clonetics). Usually, more than 85% of cells were positive for Tie2 and PECAM.

RT-PCR analysis

Total RNA was isolated from endothelial cells and unisolated cells by cell sorting (control cells) by using RNeasy Mini (Qiagen). Endothelial and control cells were isolated from E8.5 and E9.5 wild-type embryos. Reverse transcription was performed with a ThermoScript RT-PCR System (Invitrogen) in accordance with the manufacturer's instructions. PCR was performed with Ex Taq (Takara) with the use of primer sets for WAVE2, WAVE1, N-WASP and *flk-1*. RNA levels were standardized to the glyceraldehyde-3-phosphate dehydrogenase gene. PCR products were stained with ethidium bromide and quantified.

Time-lapse observation of endothelial cells

Eight thousand cells were seeded on a collagen-coated glass bottom dish (14 mm in diameter). After adhesion of cells to the dish, the medium was replaced with E-STIM endothelial culture medium containing epidermal growth factor and endothelial cell growth supplement, namely aFGF or ECGF-a (Becton Dickinson), and cultured overnight. Cells were then observed under a microscope at 10-s intervals. The sequential images were stored as a movie file of 0.2 s per frame. A VEGF gradient was made by applying VEGF (1 mg ml⁻¹ in PBS; Sigma) from a Femtotip on a microinjector (Eppendorf) at a pressure of 100 hPa. Cells that responded to VEGF were defined as cells

with any movement of cell membrane after treatment with VEGF in the movie. No significant change was observed in the absence of VEGF. The speed of membrane extension was calculated as the difference in position of the edge of the cell membrane between the start and the end of the movie.

Capillary formation

Four thousand cells were plated onto Matrigel basement membrane matrix (BD Biosciences) in one well of a 96-well plate. After culturing for 24 h in EGM-2 medium with the EGM-2 bullet kit, cells were fixed, and the area composed of capillaries was measured with NIH image software.

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Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus

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Human cytomegalovirus (HCMV) is a widespread opportunistic herpesvirus that causes severe and fatal diseases in immune-compromised individuals, including organ transplant recipients and individuals with AIDS¹. It is also a leading cause of virus-associated birth defects and is associated with atherosclerosis and

coronary restenosis¹⁻³. HCMV initiates infection and intracellular signalling by binding to its cognate cellular receptors^{4,5} and by activating several signalling pathways including those mediated by mitogen-activated protein kinase⁵⁻⁷, phosphatidylinositol-3-OH kinase⁸, interferons^{5,9}, and G proteins¹⁰. But a cellular receptor responsible for viral entry and HCMV-induced signalling has yet to be identified. Here we show that HCMV infects cells by interacting with epidermal growth factor receptor (EGFR) and inducing signalling. Transfecting EGFR-negative cells with an EGFR complementary DNA renders non-susceptible cells susceptible to HCMV. Ligand displacement and crosslinking analyses show that HCMV interacts with EGFR through gB, its principal envelope glycoprotein. gB preferentially binds EGFR and EGFR-ErbB3 oligomeric molecules in Chinese hamster ovary cells transfected with erbB family cDNAs. Taken together, these data indicate that EGFR is a necessary component for HCMV-triggered signalling and viral entry.

The EGFR family consists of four family members: EGFR (also known as erbB1 or HER1), erbB2 (HER2), erbB3 (HER3) and erbB4 (HER4)¹¹⁻¹⁵. EGFR activation initiates receptor homodimerization or hetero-oligomerization, autophosphorylation of specific tyrosine residues, receptor internalization and the induction of associated intracellular signalling¹⁵⁻¹⁸. Other family members form homodimers or hetero-oligomers with each other after activation by EGF or by the neuregulin family of ligands^{11,15,19}, providing a range of potential signalling outputs from these receptors^{12,13,15,19}. We previously observed that cells susceptible to HCMV infection express EGFR. Evidence also shows that the EGFR tyrosine kinase modulates signalling pathways originating from several receptors, including cytokine receptors, G-protein-coupled receptors and other receptor tyrosine kinases²⁰. We therefore thought that EGFR might be involved in HCMV-induced signalling and viral entry.

To determine if HCMV initiates viral signalling through EGFR, we first assessed whether HCMV infection concomitantly activated EGFR and its signal cascades. Within 5 min, HCMV addition to human embryonic lung (HEL) fibroblasts transiently activated EGFR (Fig. 1a). Phosphorylation of EGFR peaked 10 min after viral attachment and decreased to undetectable levels 2 h after infection. EGFR itself was downregulated and was undetectable 2 h after infection. The patterns of EGFR downregulation and phosphorylation during HCMV infection were similar to those observed in HEL cells treated with EGF (Fig. 1a, b). These results suggest that HCMV acts as an EGFR ligand. As described previously⁸, the phosphatidylinositol-3-OH kinase (PI(3)K)-Akt pathway was rapidly activated in HEL cells after HCMV infection. Activation of the PI(3)K pathway, including phosphorylation of the PI(3)K p85 regulatory subunit, induction of PI(3)K activity, and phosphorylation of its downstream effector Akt, was paralleled by the rapid phosphorylation of both EGFR and phospholipase C- γ (PLC- γ) and their co-precipitation (Fig. 1a).

Complete activation of another well-established target of EGFR, PLC- γ , is dependent on both tyrosine phosphorylation and translocation of the enzyme to the cytoplasmic face of the plasma membrane^{15,21}. By immunoprecipitation and immunoblot analysis, we found that PLC- γ bound to EGFR and was phosphorylated concurrently with EGFR on infection with HCMV (Fig. 1a, b). The intracellular mobilization of Ca²⁺, a downstream response of PLC- γ activation, was simultaneously activated after HCMV infection or EGF treatment (Fig. 1c). These results suggest that EGFR and its downstream signal cascades are activated on infection with HCMV. Wortmannin, a specific inhibitor of PI(3)K, inhibited the HCMV-induced activation of PI(3)K and phosphorylation of Akt without affecting the phosphorylation of EGFR (Fig. 1d), indicating that PI(3)K activation occurs downstream of HCMV-induced EGFR phosphorylation.

To assess further whether the HCMV-induced activation of PI(3)K and Ca²⁺ release are mediated through EGFR, we examined

the effect of an EGFR kinase inhibitor, AG1478, on PI(3)K activation and Ca²⁺ mobilization in HCMV-infected HEL cells (Fig. 1c, e). Activation of PI(3)K, phosphorylation of both p85 and Akt, and Ca²⁺ mobilization in HCMV-infected cells were inhibited completely by 100 nM AG1478 (Fig. 1c, left, lane 8, and 1e, lane 2). AG1478 also blocked EGF-dependent PI(3)K signalling and Ca²⁺ mobilization in HEL cells (Fig. 1e, lane 3, and 1c, right, lane 8). AG1478 did not affect the PI(3)K pathway induced by platelet-derived growth factor (PDGF; Fig. 1e, lanes 4 and 5).

We used EGFR-positive (MDA-MB468, hereafter MB468) and

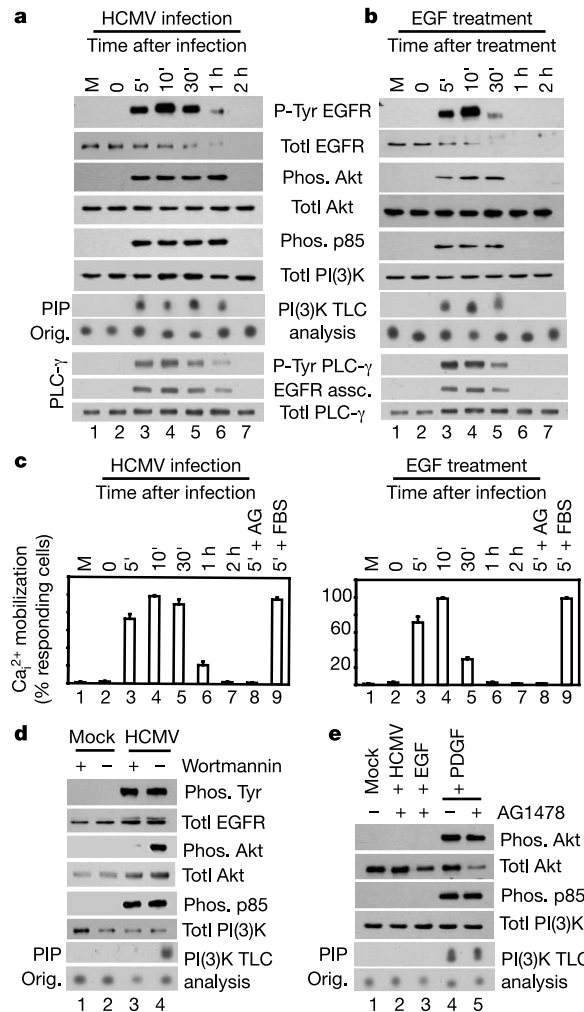


Figure 1 Activation of EGFR, PI(3)K and PLC- γ and Ca²⁺ mobilization by HCMV infection of human fibroblast HEL cells. **a–c**, Serum-starved HEL cells were infected with HCMV (**a** and **c**, left) or stimulated with EGF (**b** and **c**, right). **a**, **b**, EGFR was immunoprecipitated from lysates with an antibody against EGFR. Tyrosine-phosphorylated (P-Tyr) EGFR and PLC- γ and the phosphorylated forms of Akt and PI(3)K were detected by immunoblotting with phospho-specific antibodies. The total amounts of PI(3)K and Akt in lysates and those of immunoprecipitated EGFR, PLC- γ and PLC- γ bound to EGFR were detected by immunoblotting⁸. PI(3)K activity was determined by measuring the production of phosphatidylinositol phosphate (PIP) by thin layer chromatography (TLC)²⁹. **c**, Intracellular Ca²⁺ mobilization on HCMV infection (left) or EGF treatment (right) of HEL cells was measured using Calcium Crisom (Molecular Probes) as described²¹. Data are expressed as the percentage of cells mobilizing Ca²⁺. **d**, **e**, Effects of wortmannin (**d**) and AG1478 (**e**) on EGFR and PI(3)K activation. HEL cells were pretreated with 100 nM wortmannin or 100 nM AG1478 for 30 min before infection. Ten minutes after HCMV infection, cell lysates were analysed for phosphorylation of EGFR and activation of the PI(3)K as indicated above^{8,29}. Orig., origin; EGFR assoc., EGFR-associated PLC- γ ; AG, AG1478; FBS, 1% fetal bovine serum.

EGFR-negative (MDA-MB453, hereafter MB453) breast cancer cell lines^{22,23} to assess whether EGFR is required for HCMV infection and activation of the PI(3)K pathway (Fig. 2). HCMV did not infect or activate the PI(3)K pathway in EGFR-negative MB453 cells (Fig. 2b–e), but did both in EGFR-positive MB468 cells (Fig. 2a, c, d). Viral immediate-early (IE1, IE2), early (UL84), and late (UL94) proteins were expressed in MB468 cells after HCMV infection (Fig. 2a), but not in those treated with AG1478 (lane 7). Transfecting an EGFR expression plasmid into MB453 cells rendered them susceptible to HCMV infection (Fig. 2c–e). We confirmed both the susceptibility of MB453 cells to HCMV infection requires EGFR expression on cell surface and that infection causes internalization of EGFR (Fig. 2c). HCMV-induced activation of PI(3)K was not detected in MB453 cells transfected with a plasmid expressing a kinase-dead variant of EGFR (Fig. 2d, lane 7). These data suggest that EGFR activation is necessary for HCMV-induced PI(3)K signalling, viral entry and viral gene expression.

To assess whether HCMV directly interacts with EGFR, we pretreated HEL cells with an EGFR neutralizing antibody to block EGFR ligand binding (Fig. 3a). Pretreatment of HEL cells with the antibody blocked EGF- and HCMV-induced EGFR phosphorylation (lanes 6 and 7, respectively), but did not block PDGF-induced PDGFR receptor (PDGFR) phosphorylation (lane 8). The EGFR neutralizing antibody also inhibited the activation of PI(3)K and Akt induced by HCMV infection and by EGF treatment (Fig. 3b, lanes 3 and 4), but did not affect the activation of PI(3)K induced by PDGF (lane 5). In addition, we found that treating HEL cells with the EGFR neutralizing antibody or with AG1478 decreased the viral yield by 97% or 95%, respectively. These findings suggest that HCMV interacts with EGFR.

To address further the role of EGFR in HCMV binding and entry, purified and fluorescent *N*-hydroxysuccinimide-ester carbocyanine (Cy3)-labelled HCMV virions were used to infect MB468 and MB453 cells in the presence or absence of AG1478 or U0126.

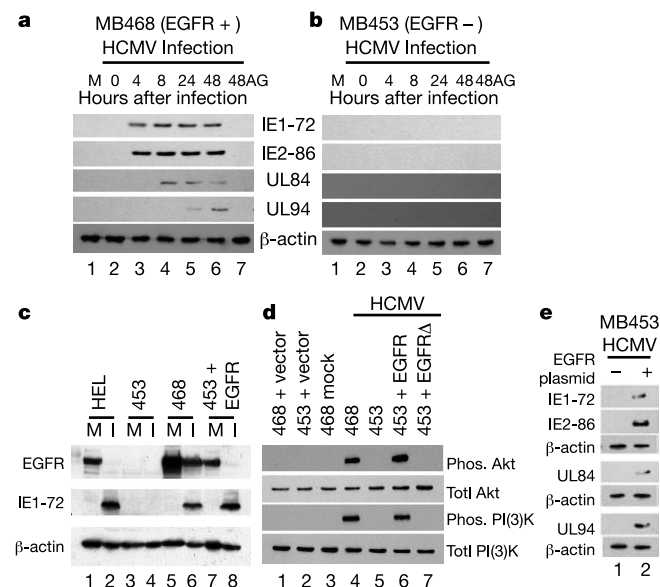


Figure 2 HCMV infection and activation of the PI(3)K kinase pathway require EGFR expression. Serum-starved MB468 (**a, c, d**), MB453 (**b, c, d, e**) and HEL (**c**) cells, and MB453 cells transiently transfected with pLXSN plasmids encoding human EGFR (453 + EGFR, **c, d**) or a kinase-dead EGFR with a mutation at Lys 721 (EGFR Δ , **d**) were infected with HCMV. Immediate-early (IE1-72 and IE2-86), early (UL84) and late (UL94) viral proteins (**a, b, c, e**), EGFR (**c**), and phosphorylated and total PI(3)K and Akt (**d**) were detected by immunoblotting. The membranes were reprobed with an antibody against β -actin to verify equal loading. AG, treated with AG1478; I, infected with HCMV; M, mock transfected.

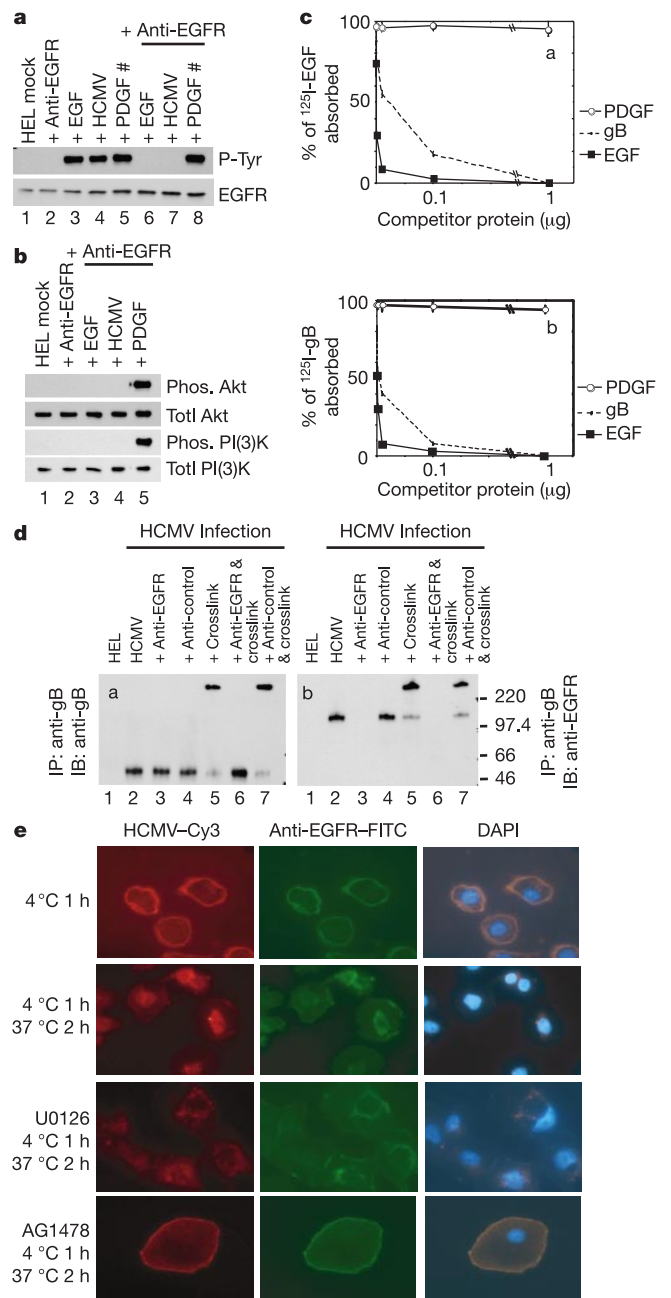


Figure 3 HCMV directly binds EGFR through gB. **a**, Serum-starved HEL cells were pretreated with an EGFR neutralizing antibody³⁰ or left untreated for 30 min before being incubated with EGF, HCMV or PDGF. After incubation, EGFR and PDGFR were immunoprecipitated and detected by immunoblotting with antibodies against EGFR, PDGFR (PDGFR#) and phosphotyrosine (P-Tyr). **b**, An EGFR neutralizing antibody blocks HCMV-induced PI(3)K signalling. HEL cell lysates were analysed for total and phosphorylated PI(3)K and Akt by immunoblotting. **c**, Ligand displacement assays. HEL cells were incubated with ¹²⁵I-labelled gB (6×10^4 c.p.m.) or ¹²⁵I-labelled EGF (1×10^5 c.p.m.) alone or with increasing concentrations of unlabelled EGF (filled squares), gB (filled circles) or PDGF (open circles). Cell-bound ¹²⁵I-labelled gB or ¹²⁵I-labelled EGF was measured. The value obtained without unlabelled competitor was considered as 100% binding. **d**, An EGFR neutralizing antibody blocks the EGFR-gB interaction. HEL cells were treated or not with an EGFR neutralizing antibody or with an unrelated control antibody (anti-control) and subsequently crosslinked to HCMV on ice with the reagent BS³. The cell lysates were immunoprecipitated with an antibody against gB and analysed by immunoblotting with antibodies against EGFR or gB. **e**, The EGFR inhibitor AG1478 blocks internalization of HCMV. MD468 EGFR-positive cells were infected with Cy3-HCMV at 4 °C, with or without subsequent incubation at 37 °C with or without AG1478 or U0126. Red fluorescence indicates Cy3-HCMV, green indicates FITC-conjugated antibodies against EGFR, and blue indicates 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained nuclei.

Infection was first carried out at 4 °C for 1 h, and then unbound virus was removed before internalization was initiated at 37 °C. We found that Cy3–HCMV bound to MB468 cell membrane at 4 °C with an image pattern similar to that of EGFR. Virus entered the cells when the subsequent incubation was carried out at 37 °C. Significantly, viral entry into MB468 cells was blocked by AG1478, an EGFR kinase-specific inhibitor. As a control, U0126, a distal downstream inhibitor of mitogen-activated protein kinase (MAPK) kinases 1 and 2 (MEK1/2) or extracellular-signal-regulated kinases 1 and 2 (ERK1/2), did not block Cy3–HCMV entry (Fig. 3e). These results indicate that the HCMV-induced activation of the EGFR intrinsic tyrosine kinase and autophosphorylation are fundamental steps for viral entry during HCMV infection. The inability of U0126 to block HCMV entry implies that the downstream MEK1/2 (or

ERK1/2) activity is not essential during the early stage of viral entry. No viral binding or entry into EGFR-negative MB453 cells was observed under similar experimental conditions (data not shown).

The HCMV principal envelope glycoprotein gB is responsible for much HCMV-induced cellular signalling^{4,9}. To identify whether HCMV binding to EGFR involves gB, we carried out receptor–ligand displacement analysis using ¹²⁵I-labelled EGF and gB (Fig. 3c). Competition between EGF and gB for binding to EGFR was observed, with displacement of ¹²⁵I-labelled EGF (Fig. 3c, top) or ¹²⁵I-labelled gB (Fig. 3c, bottom) by increasing concentrations of unlabelled gB or EGF, respectively. Unlabelled PDGF had no effect (Fig. 3c). These results indicate that gB is involved in the HCMV–EGFR interaction.

To define further this interaction, we carried out a covalent crosslinking analysis. HEL cells were incubated with purified HCMV in an ice bath for 2 h. Cells incubated with or without the crosslinking reagent bis(sulphosuccinimidyl)substrate (BS³) were lysed and immunoprecipitated with antibodies against gB or against EGFR (data not shown), and then analysed by immunoblotting with antibodies against gB or against EGFR. In the presence of BS³, gB and EGFR formed complexes in HCMV-infected cells (Fig 3d, lanes 5 and 7). This gB–EGFR interaction was disrupted by the EGFR neutralizing antibody (lanes 3 and 6) but not by a control antibody (lanes 4 and 7), suggesting that gB interacts directly with EGFR. Thus, gB seems to function as an EGFR ligand that is responsible, at least in part, for HCMV binding to EGFR.

Various EGF and neuregulin family ligands induce the formation of homodimers or hetero-oligomers of EGFR (erbB1), erbB2, erbB3 and erbB4, initiating specific intracellular signalling^{13,24}. To define further the specificity of the interaction between gB and erbB, we expressed individual as well as combinations of two erbB family members in EGFR-negative Chinese hamster ovary cells (Fig. 4). We then used crosslinking analysis to investigate the ligand–receptor interaction. ¹²⁵I-labelled EGF and gB were first incubated and then covalently crosslinked by BS³ to the various homodimeric or hetero-oligomeric erbB receptors expressed on the surface of the transfected CHO cells (Fig. 4a and Methods). gB bound to monomeric erbB1 (bottom left, lanes 1 and 5, and bottom right, lane 1) and promoted the hetero-oligomerization of erbB1 with erbB3 (bottom left, lanes 7 and 8). EGF preferentially bound to monomeric erbB1 and induced its homodimerization (top left, lanes 1, 5, and 7, and top right, lane 1) and its hetero-oligomerization with erbB2 (top left, lane 6).

We then analysed the ligand-induced phosphorylation of erbB receptors in CHO cells transfected with various erbB plasmids (Fig. 4b). EGF and gB induced tyrosine phosphorylation of EGFR (erbB1) in CHO cells transfected with an erbB1 plasmid (lanes 1 and 2). Analysis of CHO cells expressing a combination of two distinct erbB members showed that both EGF and gB preferentially induced the phosphorylation of receptors containing erbB1 (lanes 9–20). Small amounts of phosphorylated erbB proteins were also observed in EGF-treated CHO cells expressing erbB4 (lane 7), erbB2–erbB4 (lane 17) or erbB3–erbB4 (lane 19), whereas gB induced only the phosphorylation of homodimerized erbB1 or hetero-oligomerized receptors containing erbB1. This result, together with the crosslinking analysis (Fig. 3d), suggests that gB can specifically bind to and activate receptors containing erbB1. Notably, gB interacted mainly with the erbB1 monomer and the erbB1–erbB3 hetero-oligomer, whereas EGF preferred the erbB1 homodimer and erbB1–erbB2 hetero-oligomer (Fig. 4a).

To determine which subsets of erbB family proteins are required for HCMV infection, we transfected HCMV non-permissive CHO cells with individual and combinations of two erbB family cDNAs (expression was confirmed by immunoblotting; Fig. 4c, right) and then infected the cells with HCMV. erbB1 was found to be required for HCMV entry into host cells, because HCMV viral immediate-early, early and late proteins were detected only in CHO cells

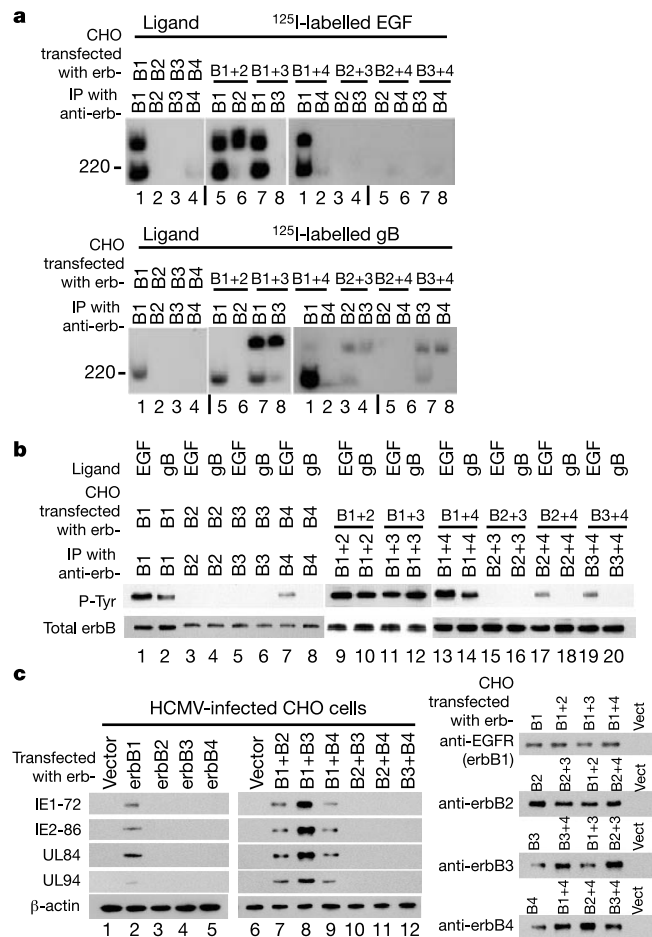


Figure 4 As an EGFR ligand, gB induces receptor phosphorylation and viral gene expression in HCMV-infected CHO cells transfected with erbB1 (B1), erbB2 (B2), erbB3 (B3) and erbB4 (B4). **a**, CHO cells were transfected with full-length cDNAs encoding individual or combinations of human EGFR family members. Cells were treated with G418 from days 2–6 after transfection. On day 6, transfected cells were crosslinked with ¹²⁵I-labelled EGF or gB. Crosslinked complexes were immunoprecipitated with antibodies against the corresponding erbB proteins, resolved on 6% SDS–PAGE gels, and detected by autoradiography. **b**, EGF and gB induce phosphorylation of EGFR. Serum starved CHO cells expressing the indicated erbBs were incubated with gB or EGF for 10 min. Cell lysates were immunoprecipitated by individual antibodies against distinct erbB proteins or combinations of two antibodies. Phosphorylated erbB (P-Tyr) and total erbB proteins were detected by immunoblotting. **c**, Viral gene expression in HCMV-infected CHO cells expressing erbB family proteins. Viral protein expression was detected by immunoblotting with the appropriate antibodies, and expression of the transfected erbB proteins was verified by immunoblotting. The membranes were reprobed with β-actin antibody to confirm equal loading.

expressing erbB1 and erbB1–erbB3 (Fig. 4c, left). The erbB1–erbB3 hetero-oligomer seemed to be the preferred combination for HCMV infection and viral gene expression. These results indicate that EGFR (erbB1) and erbB1–erbB3 can function as cellular receptors for HCMV.

HCMV initially binds to cell-surface heparan sulphate proteoglycans (HSPGs)²⁵. On their own, however, HSPGs are insufficient to render permissiveness and previous studies have indicated that more stable binding of HCMV through the engagement of a proteinaceous receptor is required^{5,25}. Major histocompatibility complex (MHC) class I molecules have been suggested as a possible receptor for HCMV, and direct interaction between HCMV gB and cellular annexin II has been reported²⁶. But none of these interactions has been linked to HCMV-induced signalling and viral entry. We have shown here that HCMV can trigger an intracellular signalling cascade and can infect target cells in a manner dependent on its interaction with EGFR. The principal HCMV envelope protein gB seems to be responsible for binding to EGFR. Although several virally encoded proteins are known to bind to EGFR²³, to our knowledge this is the first report that a viral membrane glycoprotein can function as an EGFR ligand.

HCMV infection results in a rapid decrease in EGFR expression²⁷, which implies that the interaction between HCMV and EGFR occurs at an early stage of virus and host contact. EGFR family members bind several structurally related ligands containing six covalently interacted cysteine residues¹⁴. The EGF-like domain is required for the ligand–receptor interaction. Although the ectodomain of gB molecule has 11 cysteine residues that form five disulphide bonds, we have not found a typical EGF-like domain in gB. There are, however, several stretches of sequence homology between EGF and gB molecules. Among them, the consensus sequence GX₄YX₃MEX₂HX₄Q is located immediately 3' to the EGF-like domain of EGF and in the amino-terminal region of gB molecules. It is therefore possible that gB has an epitope that can functionally mimic the EGF domain to bind to the EGFR. It will be possible to address this issue further when structural information on gB becomes available.

Our work provides direct evidence that EGFR is a necessary cellular receptor for HCMV, but it does not indicate that EGFR alone is sufficient for HCMV infection. Further investigation will be required to unravel whether other possible co-receptors are involved in viral signalling and entry, and how EGFR contributes to viral entry. It is possible that the identification of EGFR as an HCMV receptor may assist in the development of anti-HCMV agents that target the stage of viral entry. □

Methods

Virus and cells

Towne strain HCMV (p38-40) was passaged, purified and titrated as described⁸. We obtained HEL fibroblasts, CHO cells and human breast cancer cells MB453 and MB468 from the American Type Culture Collection and cultured them as described^{8,22}. Cells were transfected by the calcium phosphate method.

Immunoprecipitation and immunoblotting

Cells were extracted with a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 20 μ M leupeptin and 1.5 μ M aprotinin. We determined the protein concentration in the cell lysate by a protein assay kit (Bio-Rad). Immunoprecipitation and immunoblot analysis were done as described⁹.

Cy3 labelling, and viral binding and internalization

Purified extracellular HCMV virions were labelled with Cy3 using the Fluoro-Link antibody Cy3 labelling kit (Amersham) according to the manufacturer's instructions. We carried out virus binding and internalization assays, and immunofluorescence as described²⁸. EGFR was detected by an indirect immunofluorescence test with a fluorescein isothiocyanate (FITC)-labelled secondary antibody. Infected and control cells on chamber slides were mounted in Mowiol (Aldrich) and examined with an epifluorescence microscope (Zeiss Axioskop). We analysed images with the Openlab 3.0.7 imaging system (Zeiss).

Ligand displacement and covalent crosslinking

Ligands were labelled with ¹²⁵I (Amersham) to a specific activity of 1×10^5 c.p.m. per ng by Iodogen (Pierce) according to the manufacturer's instructions. For ligand displacement analysis, cells were plated in six-well plates and incubated for 2 h at 4 °C with radiolabelled ligand and various concentrations of unlabelled ligands. The labelled cells were then washed and lysed, and radioactivity was determined using a γ -counter. For the crosslinking analysis, 2×10^5 cells were incubated for 2 h on ice with radiolabelled ligand (1×10^7 c.p.m. per ml) or unlabelled HCMV at multiplicity of infection of 100 p.f.u. per cell. We added the chemical crosslinking reagent BS³ (Pierce) to a concentration of 1 mM. After 45 min at 4 °C, cells were lysed and the lysates immunoprecipitated using antibodies against gB or the four members of the erbB family. In some experiments, the cells were pretreated before crosslinking with an EGFR neutralizing antibody (#05101, clone A1, Upstate Biotech). The crosslinked complexes or immunoprecipitation products were resolved on 6% SDS–PAGE gels and detected directly by autoradiography or immunoblot analysis.

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RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo

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The small GTPase Rac has a central role in regulating the actin cytoskeleton during cell migration and axon guidance¹. Elmo has been identified as an upstream regulator of Rac1 that binds to and functionally cooperates with Dock180 (refs 2–4). Dock180 does not contain a conventional catalytic domain for guanine nucleotide exchange on Rac, but possesses a domain that directly binds to and specifically activates Rac1 (refs 5, 6). The small GTPase RhoG mediates several cellular morphological processes, such as neurite outgrowth in neuronal cells, through a signalling cascade that activates Rac1 (refs 7–12); however, the downstream target of RhoG and the mechanism by which RhoG regulates Rac1 activity remain unclear. Here we show that RhoG interacts directly with Elmo in a GTP-dependent manner and forms a ternary complex with Dock180 to induce activation of Rac1. The RhoG–Elmo–Dock180 pathway is required for activation of Rac1 and cell spreading mediated by integrin, as well as for neurite outgrowth induced by nerve growth factor. We conclude that RhoG activates Rac1 through Elmo and Dock180 to control cell morphology.

RhoG shares high amino acid sequence identity with Rac1 and Cdc42 (72% and 62%, respectively), but does not bind to known Rac1 and Cdc42 effectors, including proteins containing a Cdc42/Rac interactive binding (CRIB) motif⁷. To isolate potential effectors for RhoG, we carried out a yeast two-hybrid screen of a rat brain complementary DNA library using a constitutively active mutant RhoG (RhoG-V12) as a bait. We isolated several positive clones, one of which encoded full-length rat Elmo2 (98.6% amino acid identity to human Elmo2).

To determine whether the interaction of RhoG with Elmo was dependent on GTP, full-length Elmo tagged with haemagglutinin A (HA) was expressed in 293T cells and used in a pull-down assay with purified glutathione S-transferase (GST)-fused RhoG preloaded with GDP or GTP- γ S. Elmo interacted with GTP-bound active RhoG but not with GDP-bound inactive RhoG or GST alone (Fig. 1a). Using deletion mutants of Elmo, we identified the amino-terminal region of Elmo (amino acids 1–362, Fig. 1e) as sufficient for the interaction with RhoG (Fig. 1a).

To examine whether Elmo interacts specifically with RhoG among the Rho family GTPases, Myc-tagged constitutively active RhoG (RhoG-V12) and other well-characterized Rho family

GTPases were expressed and used in a pull-down assay with the purified GST-fused N-terminal region of Elmo (Elmo-NT). RhoG-V12 strongly bound to Elmo-NT, whereas constitutively active Rac1 (Rac1-V12), Cdc42 (Cdc42-V12) and RhoA (RhoA-V14) did not, although Rac1-V12 and Cdc42-V12 interacted with the GST-fused CRIB domain of Pak, and RhoA-V14 interacted with the Rho-binding domain of Rhotekin (refs 13, 14 and Fig. 1b). A dot-blot assay using GTP- γ S- or GDP-loaded purified GST–RhoG as a probe showed that the GTP-bound form of RhoG directly interacted with Elmo (Fig. 1c). The interaction between active RhoG and Elmo also occurred in mammalian cells, as observed by immunoprecipitation studies of 293T cells co-transfected with HA-tagged full-length Elmo and Myc-tagged RhoG-V12 (Fig. 1d). By contrast, RhoG-V12 containing a Phe37Ala substitution in the effector region (RhoG-V12A37), which has no ability to cause morphological changes^{8–10}, or a dominant-negative variant of RhoG (RhoG-N17) did not bind to Elmo (Fig. 1d).

Because Elmo interacts with Dock180 through its carboxy-terminal region^{4,5}, we tested whether active RhoG could form a ternary complex with Elmo and Dock180 by a co-immunoprecipitation study in 293T cells co-transfected with Flag–Dock180, HA–Elmo and Myc–RhoG-V12. We did not detect an interaction between Dock180 and RhoG-V12 in the absence of Elmo; however, Dock180 was co-immunoprecipitated with RhoG-V12 when the two proteins were co-expressed with Elmo (Fig. 2a). RhoG-V12A37, which had no ability to bind Elmo, did not precipitate Dock180. In

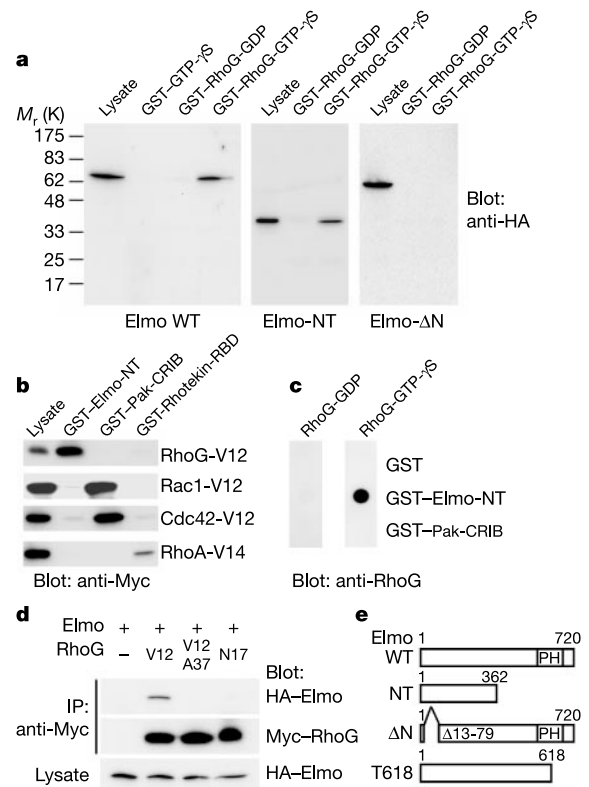


Figure 1 Elmo binds directly to RhoG in a GTP-dependent manner. **a**, HA-tagged variants of Elmo (wild type (WT), NT, Δ N) expressed in 293T cells were used in a pull-down assay with GST–RhoG preloaded with GDP or GTP- γ S. Bound proteins were analysed by immunoblot analysis. **b**, Myc-tagged constitutively active Rho GTPases were used in a pull-down assay with the indicated GST fusion proteins. **c**, The indicated GST proteins were probed with GST–RhoG loaded with GDP or GTP- γ S. Bound GST–RhoG was detected with an antibody against RhoG. **d**, Cell lysates transfected with the indicated plasmids were immunoprecipitated with an antibody against Myc. **e**, The Elmo constructs used in this study. PH, pleckstrin homology domain.

addition, when Dock180 and RhoG-V12 were co-transfected with a mutant of Elmo lacking the C-terminal 100 amino acids necessary for binding to Dock180 (Elmo-T618)⁵, Dock180 was not precipitated with RhoG-V12, although an interaction between Elmo-T618 and RhoG-V12 was observed (Fig. 2a). These results indicate that Elmo mediates the interaction between active RhoG and Dock180.

Elmo and Dock180 localize mainly in the cytoplasm, but the recruitment of Dock180 to the plasma membrane is thought to be an important step for Dock180-mediated reorganization of the cytoskeleton^{2,15,16}. To determine whether RhoG regulates the subcellular localizations of Elmo and Dock180, we prepared crude membrane and cytosolic fractions from cellular homogenates of 293T cells expressing HA-Elmo and Flag-Dock180 and analysed their distributions by immunoblot analysis. When cells were transfected with Dock180 alone or together with Elmo, they were observed largely in the cytosolic fraction. By contrast, Dock180 and Elmo were detected in the membrane fraction when they were co-expressed with RhoG-V12, although RhoG-V12A37 had no effect (Supplementary Fig. 1a). In addition, when Dock180 was coexpressed with Elmo-T618 and RhoG-V12, Elmo-T618 was detected in the membrane fraction but Dock180 was not.

To examine further the effect of RhoG on the subcellular localizations of Elmo and Dock180 by immunofluorescence analysis, Elmo fused to green fluorescent protein (GFP) was co-expressed with Flag-Dock180 in HeLa cells. When cells were transfected with GFP-Elmo and Flag-Dock180, the proteins localized in the cytoplasm. By contrast, when cells were co-transfected with RhoG-V12, some GFP-Elmo and Flag-Dock180 proteins were localized at the plasma membrane and were clearly co-localized with RhoG-V12 (Supplementary Fig. 1b). Co-expression of Rac1-V12 had no effect on the localization of GFP-Elmo and Flag-Dock180 (data not shown). These results indicate that the interaction of RhoG with Elmo induces translocation of the Elmo-Dock180 complex to the plasma membrane. Although we could not obtain antibodies for detecting endogenous Elmo, endogenous Dock180 was co-

immunoprecipitated with RhoG-V12 and was recruited to the membrane fraction (Supplementary Fig. 2).

Because Dock180 directly binds to and exchanges GDP for GTP on Rac1 (refs 5, 6), we tested whether active RhoG might modulate the activity of Dock180 toward Rac1 in the presence of Elmo in cells by using the GST-fused CRIB domain of Pak to precipitate GTP-bound active Rac1 from the cell lysates. As reported previously⁵, expression of Dock180 alone in 293T cells induced an increase in Rac1 activity, and co-expression of Elmo enhanced this Dock180-mediated activation of Rac1. Furthermore, expression of RhoG-V12 increased the Dock180- and Elmo-mediated activation of Rac1, as well as the basal Rac1 activity, about twofold (Fig. 2b). This effect was not observed when RhoG-V12A37 was expressed instead of RhoG-V12. In addition, Elmo-T618, which does not interact with Dock180, completely blocked the RhoG-V12-induced augmentation of Dock180 activity towards Rac1 (Fig. 2b). These results indicate that RhoG promotes the Dock180-mediated activation of Rac1 through Elmo.

To determine whether Elmo and Dock180 function downstream of RhoG, we co-expressed RhoG-V12 with either wild-type Elmo or the Elmo-T618 mutant in HeLa cells. As observed previously in fibroblasts^{7,8}, expression of RhoG-V12 in HeLa cells induced membrane ruffling (Fig. 2c), and a dominant-negative variant of Rac1 (Rac1-N17) suppressed this effect (data not shown). Whereas co-expression of wild-type Elmo with RhoG-V12 had little effect on the RhoG-V12-induced cytoskeletal change, co-expression of Elmo-T618 significantly suppressed the RhoG-V12-induced membrane ruffling (Fig. 2c and Supplementary Fig. 3a), suggesting that the interaction of Elmo with Dock180 is required for the RhoG-V12-induced membrane ruffling.

We prepared a mutant of Dock180 that binds to Elmo but does not interact with or activate Rac1 (Dock180-ISP, in which Ile-Ser-Pro is mutated to Ala-Ala-Ala at amino acids 1487–1489)⁵. Expression of Dock180-ISP inhibited the RhoG-V12-induced membrane ruffling, whereas wild-type Dock180 had no effect

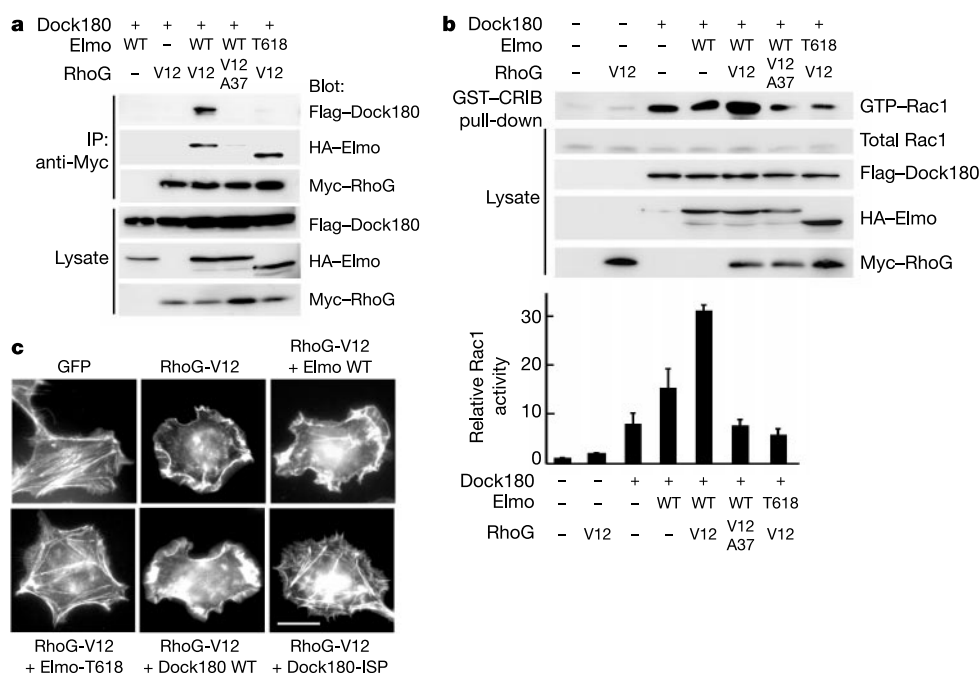


Figure 2 RhoG activates Rac1 through Elmo and Dock180. **a**, Cell lysates from 293T cells transfected with the indicated plasmids were immunoprecipitated with an antibody against Myc. **b**, Cell lysates from 293T cells transfected with the indicated plasmids were incubated with GST-CRIB, and bound Rac1 was detected with an antibody against Rac1.

Relative Rac1 activity was determined by the amount of Rac1 bound to GST-CRIB normalized to the amount of Rac1 in cell lysates analysed by NIH Image software. Results are the means $\pm$ s.e.m. of triplicate experiments. **c**, HeLa cells were transfected with the indicated plasmids and stained for F-actin. Scale bar, 20 μ m.

(Fig. 2c and Supplementary Fig. 3a). These inhibitory effects of Elmo-T618 and Dock180-ISP were specific to RhoG, because expression of Elmo-T618 or Dock180-ISP had no effect on the Rac1-V12-induced membrane ruffling (Supplementary Fig. 3a).

Some cells expressing Dock180-ISP produced actin microspikes (Fig. 2c), and a similar cytoskeletal change was observed in cells expressing Rac1-N17 (data not shown). These microspikes were probably induced by activation of Cdc42 by RhoG-V12 as reported previously^{7,8}. The formation of microspikes was not observed in cells expressing Elmo-T618, which may imply that Elmo-T618 and Dock180-ISP suppress RhoG-V12-mediated signalling at different points. We also confirmed the involvement of Elmo in the RhoG-V12-induced cytoskeletal change by co-transfecting cells with RhoG-V12 and an Elmo-specific short interfering RNA (siRNA) expression vector, which effectively reduced the amounts of endogenous Elmo messenger RNA (Supplementary Fig. 3b, c). These results show that RhoG regulates the actin cytoskeleton through Rac1 activation mediated by Elmo–Dock180, and that Elmo-T618 and Dock180-ISP act as dominant-negative forms of the proteins.

Previous studies have identified Dock180 as an essential downstream component of integrin signalling^{16–19}. To investigate the role of RhoG and Elmo in the downstream signalling pathway of integrin, we plated HeLa cells onto fibronectin and observed their morphological changes by staining for F-actin. As shown previously in fibroblasts^{20,21}, HeLa cells rapidly adhered to fibronectin, and by 15 min began to spread and showed extensive membrane ruffling at

the cell periphery. These morphological changes were suppressed by the expression of a dominant-negative variant of RhoG (RhoG-N17), Elmo-T618 or Dock180-ISP (Fig. 3a). We also observed that plating 293T cells on fibronectin stimulated Rac1 activity, which was inhibited by expression of Elmo-T618 or Dock180-ISP (Fig. 3b). These results suggest that the RhoG–Elmo–Dock180 pathway is crucial in the integrin-mediated activation of Rac1 and cell spreading.

To determine whether Elmo and Dock180 are also involved in RhoG-mediated neurite outgrowth, PC12 cells were transfected with Elmo-T618 or Dock180-ISP, and the morphology of transfected cells was examined after stimulation with nerve growth factor (NGF). Similar to the expression of a dominant-negative variant of RhoG (RhoG-N17), overexpression of Elmo-T618 or Dock180-ISP strongly inhibited the NGF-induced neurite outgrowth in PC12 cells (Fig. 4a). In addition, Elmo-ΔN, which does not associate with RhoG (Fig. 1a) but retains the ability to interact with Dock180 (refs 2, 4), was as effective as Elmo-T618 in blocking neurite outgrowth (Fig. 4a). We also found that these dominant-negative forms of Elmo and Dock180 blocked RhoG-induced neurite outgrowth (Fig. 4b). These results suggest that Elmo and Dock180 are involved in NGF signalling downstream of RhoG, leading to neurite outgrowth.

Our results identify Elmo as a specific target of RhoG that mediates Rac1 activation and show that RhoG is an important upstream regulator of Rac1. On the basis of our results and previous reports that RhoG is a target GTPase of Trio or the Trio-related rhoGEF Kalirin^{8,10,11}, we propose that the Trio/Kalirin–RhoG–Elmo–Dock180 pathway is a previously unknown pathway

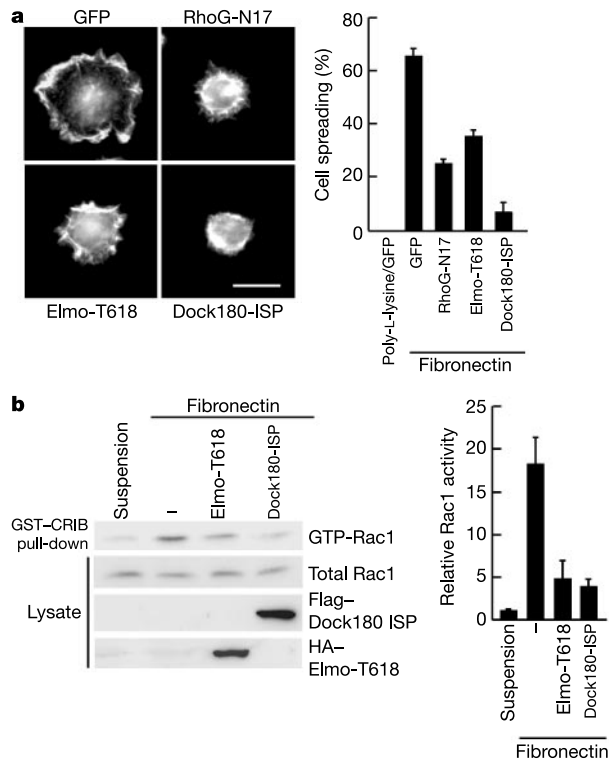


Figure 3 Mutants of Elmo and Dock180 suppress Rac1 activation and cell spreading mediated by integrin. **a**, HeLa cells transfected with the indicated plasmids were incubated with trypsin, replated on fibronectin-coated coverslips for 15 min, and then stained for F-actin. Cells that had fully spread were scored as a percentage of the total number of transfected cells, and results are the mean $\pm$ s.e.m. of triplicate experiments in which 50 cells were counted. **b**, Lysates from 293T cells expressing the indicated proteins and cultured on fibronectin-coated plates for 15 min were incubated with GST–CRIB, and bound Rac1 was detected with an antibody against Rac1. Relative Rac1 activity was determined as described in the legend to Fig. 2b.

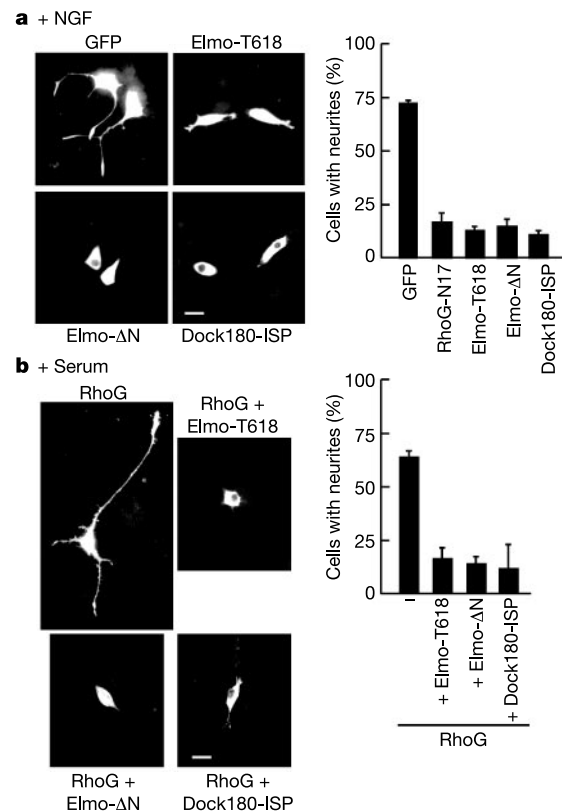


Figure 4 Mutants of Elmo and Dock180 suppress NGF- and RhoG-induced neurite outgrowth in PC12 cells. **a**, Cells were transfected with the indicated plasmids and stimulated with NGF (100 ng ml⁻¹) for 48 h. **b**, Cells were transfected with the indicated plasmids and cultured in growth medium for 48 h. Scale bar, 20 μ m. In **a** and **b**, cells with neurites were scored as a percentage of the total number of transfected cells, and results are the mean $\pm$ s.e.m. of triplicate experiments in which 50 cells were counted.

for regulating Rac1 activity during neurite outgrowth and other cellular morphological events. Because cell migration and axon outgrowth in *Caenorhabditis elegans* involves the Elmo and Dock180 homologues CED-12 and CED-5, as well as the Trio homologue UNC-73 (refs 22, 23), we speculate that MIG-2, which is a target GTPase of UNC-73 and is also involved in cell migration and axon outgrowth^{22–24}, may mediate the signalling from UNC-73 to CED-12. □

Methods

Expression plasmids and antibodies

Plasmids expressing Myc-tagged RhoG-V12, RhoG-V12A37, RhoG-N17, RhoA-V14, Rac1-V12 and Cdc42-V12 were generated pEF-BOS as described^{9,25}. The expression plasmid encoding Flag-tagged DOCK180 was a gift from M. Matsuda. Rat Elmo2 and its deletion mutants were subcloned into pCMV-HA (Clontech). The siRNA for Elmo was designed to target 19 nucleotides after the two adenine residues at nucleotides 105 and 106 of the human Elmo2 transcript, and was expressed by using an siRNA expression vector (Ambion). We used the following antibodies: mouse monoclonal antibodies against Myc and against Dock180, and rabbit polyclonal antibodies against HA and against RhoG (Santa Cruz Biotechnology); a rat monoclonal antibody against HA (Roche); a mouse monoclonal antibody against Rac1 (Transduction Laboratories); a mouse monoclonal antibody against Flag (Sigma); secondary antibodies conjugated to horseradish peroxidase (DAKO); and secondary antibodies conjugated to Alexa 488 or 594 (Molecular Probes).

Cell culture and transfection

PC12, 293T and HeLa cells were transfected with Lipofectamine 2000, Lipofectamine Plus (both Invitrogen) and Superfect (Qiagen), respectively, according to the manufacturer's instructions. For integrin stimulation, cells were incubated with trypsin and suspended in serum-free medium containing 1 mg ml⁻¹ trypsin inhibitor. After 1 h, the cells were plated on 10 µg ml⁻¹ fibronectin-coated plates blocked with 1% bovine serum albumin plates or coverslips. For the neurite outgrowth assay, we seeded PC12 cells onto glass coverslips coated with poly-D-lysine and differentiated them with 100 ng ml⁻¹ NGF for 48 h. Cells with neurites were defined as cells that possessed at least one neurite longer than the twice the diameter of the cell body.

Pull-down assay and immunoprecipitation

Purification of recombinant GST fusion proteins from *Escherichia coli*, *in vitro* binding assays, and immunoprecipitation were done as described²¹. We loaded recombinant GST-RhoG with guanine nucleotides by incubating the protein with 100 µM GTP-γS or GDP in loading buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol and 5 mM EDTA) at 30 °C for 10 min. The reaction was stopped by adding MgCl₂ to final concentration of 10 mM. Activation of Rac1 in cells was determined by using the GST-fused CRIB domain of Pak1 as described⁹.

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Crystal structure of human cytochrome P450 2C9 with bound warfarin

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Cytochrome P450 proteins (CYP450s) are membrane-associated haem proteins that metabolize physiologically important compounds in many species of microorganisms, plants and animals. Mammalian CYP450s recognize and metabolize diverse xenobiotics such as drug molecules, environmental compounds and pollutants¹. Human CYP450 proteins CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are the major drug-metabolizing isoforms, and contribute to the oxidative metabolism of more than 90% of the drugs in current clinical use². Polymorphic variants have also been reported for some CYP450 isoforms, which has implications for the efficacy of drugs in individuals, and for the co-administration of drugs. The molecular basis of drug recognition by human CYP450s, however, has remained elusive. Here we describe the crystal structure of a human CYP450, CYP2C9, both unliganded and in complex with the anti-coagulant drug warfarin. The structure defines unanticipated interactions between CYP2C9 and warfarin, and reveals a new binding pocket. The binding mode of warfarin suggests that CYP2C9 may undergo an allosteric mechanism during its function. The newly discovered binding pocket also suggests that CYP2C9 may simultaneously accommodate multiple ligands

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during its biological function, and provides a possible molecular basis for understanding complex drug–drug interactions.

So far the understanding of the molecular basis of drug binding by human CYP450s has been derived largely from the structures of bacterial CYP450s complexed to ligands^{3–6}; however, the low amino acid sequence identity (20–30%) between the human drug-metabolizing and the bacterial CYP450s limits this approach. Furthermore, despite the report of the crystal structure of a mammalian CYP450, CYP2C5 from rabbit⁷, insights into how the human CYP450s are able to recognize structurally diverse ligands remain elusive. In this study we report the crystal structure of a human cytochrome P450, CYP2C9. The structure of the protein has been determined in the absence and presence of a known substrate, the anti-coagulant drug warfarin.

CYP2C9 is a two-domain protein with an overall fold characteristic of the CYP450 family (Fig. 1a). The B–C loop contributes to substrate specificity^{8,9}, and in both the substrate-free and complexed structures of CYP2C9 residues 101–106 in the B–C loop form helix B'. In addition, residues 212–222 in the F–G loop form helices F' and G', a feature previously not observed in any other CYP450 structure. The haem is located between helices I and L and the iron is pentacoordinated with Cys 435 as the single ligand, and despite the lack of an ordered water molecule in the sixth coordination position, the spin state of the haem iron in the crystal is unknown, as the environment may change during the experiment. As in other CYP450 structures, a water molecule, located 7 Å above the haem iron (Supplementary Fig. S1), is hydrogen bonded to a highly conserved threonine, Thr 301, appropriate for its role in the proton-transfer path¹⁰. In addition there is some residual electron density located 4 Å above the haem, running up to and alongside helix I (Supplementary Fig. S1). The features of this electron density make an interpretation ambiguous; however, it does not appear close enough to the haem iron to be a sixth ligand. The haem is stabilized by hydrogen bonds between the propionates and the side chains of residues Trp 120, Arg 124, His 368 and Arg 433. A key

residue implicated by mutagenesis studies¹¹, Arg 97, also forms hydrogen bonds to the propionates, as well as the carbonyl oxygen atoms of Val 113 and Pro 367 (Fig. 1b). Thus it would seem that the main role of Arg 97 in CYP2C9 is haem stabilization rather than substrate interaction as previously suggested^{11,12}.

Several reports indicate that CYP2C9 has a preference for small acidic lipophilic compounds as substrates, implying the presence of basic residues within the protein active site, and leading to the hypothesis of an 'anionic-binding site'^{12–14}. However, although a number of hydrophobic residues are within the active site cavity, there appear to be no basic residues with the potential to interact with substrates. The active site cavity extends up and away from the I helix, with Phe 114 and Phe 476 lying on opposite sides of the channel, and the very top of the channel being formed by the B' helix, and the B–C and F–G loops. Phe 114 points into the active site and is well positioned to form interactions with substrates as implicated by mutagenesis⁸. Residues Phe 69, Phe 100, Leu 102, Leu 208, Leu 362, Leu 366 and Phe 476 form a hydrophobic patch in the active site whereas Arg 105 and Arg 108, previously implicated in the formation of the putative anionic-binding site, both point away from the cavity. In contrast to basic residues, there are two acidic residues present in the active site of CYP2C9. Asp 293 is close to Phe 110 and Phe 114, and forms a hydrogen bond to the backbone nitrogen of Ile 112 and consequently is well ordered, whereas Glu 300 points into the active site but shows a degree of flexibility in the substrate-free structure. Furthermore, Gln 214 and Asn 217 are both close to Phe 476 and could offer potential hydrogen-bonding interactions with ligands. The human isoforms CYP2C9 and CYP2C19 differ by 43 residues out of 490, and of these there is only one non-conservative substitution within the active site: residue 99 is an isoleucine in CYP2C9 and a histidine in CYP2C19. However, unlike CYP2C9, CYP2C19 shows no apparent preference for compounds containing an acidic group¹⁵. The widely held view is that this difference in the substrate selectivity for these two isoforms is due to the nature of the amino acids within their

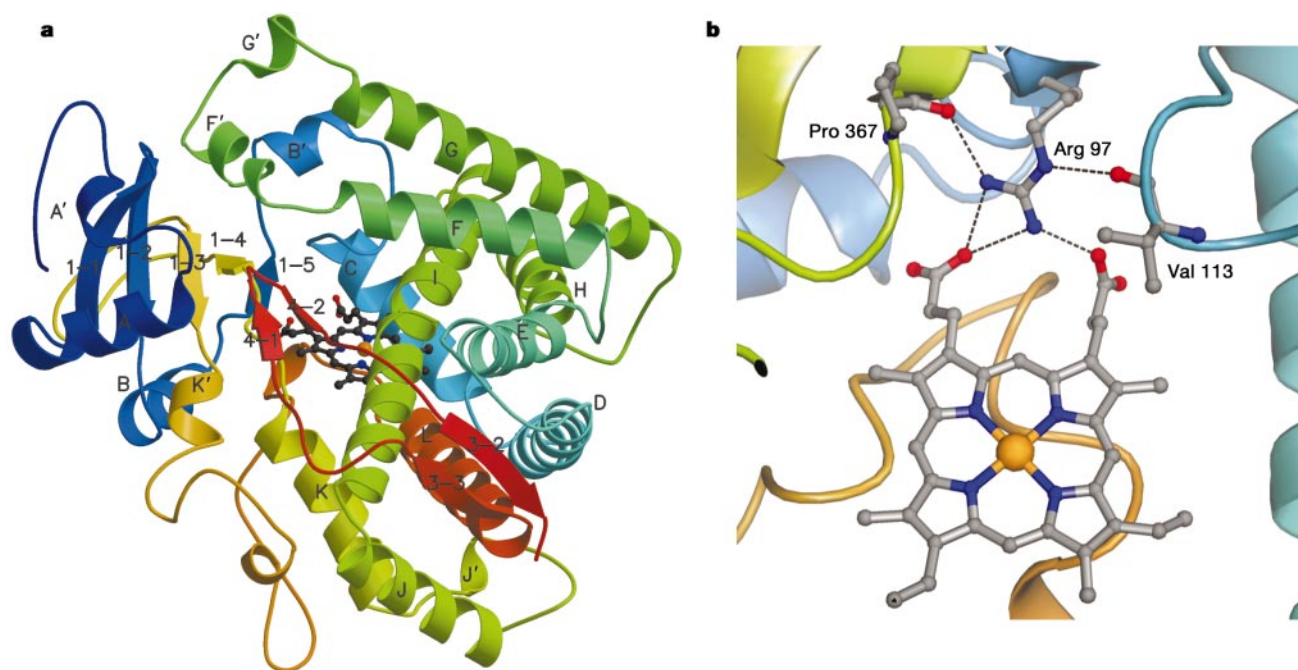


Figure 1 Structure of P450 CYP2C9. **a**, Overall fold of CYP2C9, coloured from blue at the N terminus to red at the C terminus. The haem group is depicted as a ball-and-stick model in the centre of the molecule, flanked by helices I and L. There is a slight distortion in helix I, close to the haem. The substrate access channel is widely acknowledged to involve the loops between helices B and C, and helices F and G. The figure was produced using

Molscript (<http://www.avatar.se/molscript>). **b**, View of Arg 97 and the haem group (shown at the bottom). Arg 97 is held in position by hydrogen bonds (indicated by dashed lines) to the haem propionates and to the carbonyl oxygen atoms of Val 113 and Pro 367. Figures 1b–4b were produced using Aesop 2.5 (M. Noble, unpublished work).

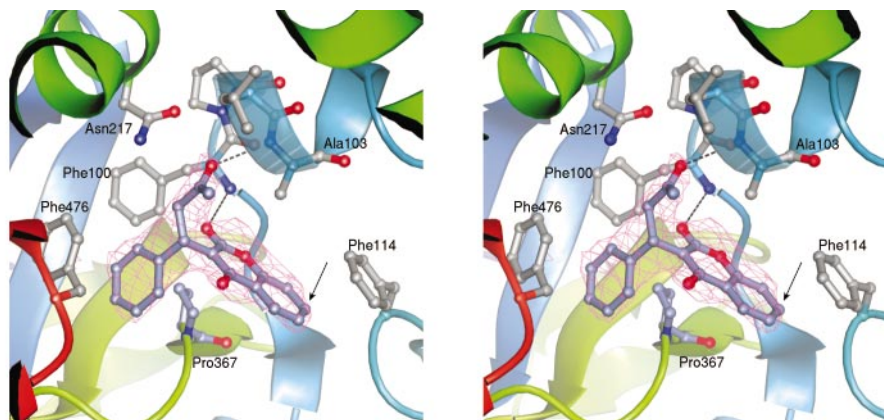


Figure 2 Stereo view of the binding site of *S*-warfarin in CYP2C9. The initial sigma-weighted $F_o - F_c$ electron density map is shown in red at the 2.5 sigma level. The hydrogen bonds between *S*-warfarin and the backbone nitrogen amide atoms of Phe 100 and Ala 103 are depicted as dashed lines. The pi-pi stacking interactions between the

phenyl group of *S*-warfarin and Phe 476 is shown, as is the van der Waals contact between the bicyclic template of *S*-warfarin and residues Ala 103, Phe 114 and Pro 367. An arrow indicates the site of the 7-hydroxylation of *S*-warfarin catalysed by CYP2C9.

active sites. With the absence of basic residues in the active site of CYP2C9, the selectivity of these proteins may lie elsewhere, such as the substrate-access channel in which Lys 72 in CYP2C9 is substituted with a glutamate in CYP2C19.

CYP2C9 catalyses the 6- and 7-hydroxylation of the active enantiomer of warfarin, *S*-warfarin, to inactive metabolites^{16,17}. To explore the molecular basis of drug binding we have determined the crystal structure of CYP2C9 complexed with *S*-warfarin. *S*-warfarin lies in a predominantly hydrophobic pocket lined by residues Arg 97, Gly 98, Ile 99, Phe 100, Leu 102, Ala 103, Val 113, Phe 114, Asn 217, Thr 364, Ser 365, Leu 366, Pro 367 and Phe 476. More specifically, the phenyl group of *S*-warfarin packs against the side chains of Phe 476 and Phe 100 and also contacts Pro 367 (Fig. 2). Although the binding of *S*-warfarin to CYP2C9 appears not to induce major conformational changes within the protein, some local rearrangements are observed. For example, the presence of the compound has slightly displaced the loop containing residues 474–478 by 0.5–1.5 Å. The side chain of Phe 476, which showed conformational mobility in the substrate-free structure, forms a pi-pi stacking interaction with the *S*-warfarin phenyl group. The bicyclic scaffold of *S*-warfarin also makes van der Waals contact with the side chains of Ala 103, Phe 114 and Pro 367 (Fig. 2). Hydrogen bonding interactions are observed between carbonyl oxygen atoms of the warfarin and backbone amide nitrogen atoms of Phe 100 and Ala 103 (Fig. 2). The position of the warfarin molecule places the potential site of hydroxylation about 10 Å from the haem iron (Fig. 3). This is consistent with spectroscopic analysis performed in solution, which detected only a small increase in the high spin content of the oxidized protein when *S*-warfarin was added, indicating that the haem is unperturbed.

Although many of the residues lining the warfarin-binding pocket have been shown by mutagenesis to alter the catalytic properties of CYP2C9 (refs 9, 18), this region had not been previously identified as a ligand-binding site. The large active site (approximately 470 Å³) and the lack of significant conformational change on compound binding raises the intriguing possibility for additional small molecules to simultaneously bind within the active site. When bound in this orientation and location, the *S*-warfarin molecule is believed to be too distant from the hydroxylation to occur, suggesting that the compound moves from this primary recognition site towards the haem to facilitate catalysis. We speculate that such a two-step conformational movement may be triggered by an electron-transfer-driven conformational change within CYP2C9, perhaps on reduction of the haem iron or interaction with the electron-transfer partner, cytochrome P450 reductase^{19,20}. The

redox state of the iron is unknown; the starting material used for crystallization was oxidized protein, but we cannot discount partial reduction of the haem iron by X-rays during data collection²¹. It is also possible that some ligands that bind in the warfarin-binding pocket are unable to move closer to the haem and could behave as competitive inhibitors by occupying this binding site. With *S*-warfarin bound in this position, there is space for a second drug molecule to bind at the haem—molecular modelling indicates that either another molecule of *S*-warfarin (Fig. 4a) or a different molecule, such as fluconazole (Fig. 4b), could simultaneously bind.

The discovery of this new warfarin-binding site may have implications for understanding the complex mechanisms used by the human CYP450 proteins during their function. There are many reports that the human CYP450 proteins catalyse reactions exhibit-

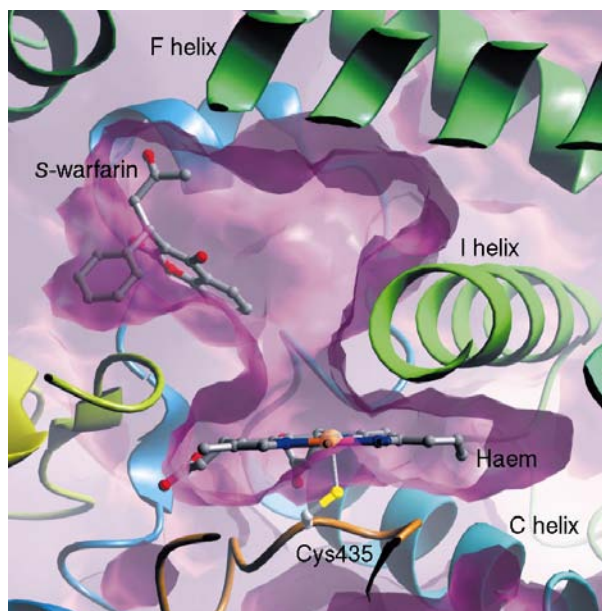


Figure 3 The surface of the active site of CYP2C9. The haem is shown at the bottom, and the *S*-warfarin molecule above and to the left. The active site of CYP2C9 is large and could potentially accommodate either compounds larger than *S*-warfarin or multiple ligands of comparable size to *S*-warfarin without substantial conformational movement. With *S*-warfarin bound in this location the haem group remains available to metabolize additional substrate molecules.

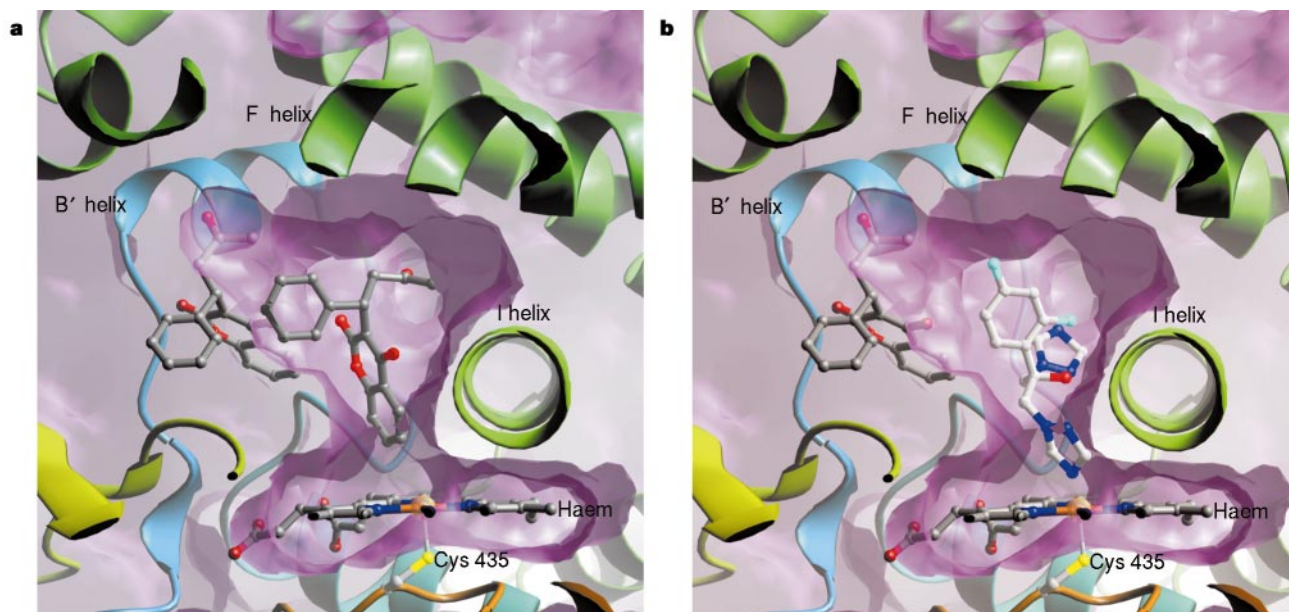


Figure 4 View of the region of the active site of CYP2C9 that remains available to accommodate additional ligand(s) after *S*-warfarin. The bound *S*-warfarin molecule is shown as in Fig. 3. **a**, A second molecule of *S*-warfarin has been modelled into the active

with the site of hydroxylation closest to the haem iron. **b**, A known haem binder fluconazole has been modelled into the cavity in a similar conformation to that observed in the complex of CYP51 with fluconazole (Protein Data Bank code 1EA1).

ing atypical kinetics such as activation, auto-activation and substrate inhibition²². Most of the observations citing this cooperativity have been made with CYP3A4, which routinely exhibits a capacity for multiple ligand binding during its function²³. The crystal structure indicates that CYP2C9 may also have the capacity to bind multiple substrates/ligands simultaneously during its function and is consistent with reports that implicate a 'two-site model' for CYP2C9 (refs 24, 25). The warfarin-binding site could be one of these sites which, when occupied, 'activates' CYP2C9 through an allosteric mechanism, as there is sufficient space for other substrate molecules to bind at the haem (Figs 3 and 4). This is consistent with data that show that CYP2C9 can be activated by some compounds^{24,25} including *S*-warfarin, which increases the rate of metabolism of 7-methoxy-4-trifluoromethylcoumarin²⁶. However the metabolism of *S*-warfarin itself by CYP2C9 follows Michaelis-Menten kinetics²⁷, indicating that no auto-activation occurs. Furthermore, although the phenomenon of CYP450-mediated drug-drug interactions is widely reported for many drugs, the molecular basis remains unclear. A drug molecule bound at the warfarin-binding site would be ideally placed to make direct molecular interactions with another drug molecule interacting with the haem group. Whether this binding site is a 'primary binding site' for substrates, an 'inhibitor-binding site', or has a role in an allosteric mechanism and drug-drug interactions, will be the focus of further investigation. □

Methods

Protein expression, purification and characterization

The amino-terminal transmembrane domain of CYP2C9 (residues 1–29) was replaced by the short charged, hydrophilic polypeptide MAKKTSSKGR, and a four-histidine tag was introduced at the carboxy terminus to facilitate solubilization and protein purification. Seven amino acid substitutions (Lys206Glu, Ile215Val, Cys216Tyr, Ser220Pro, Pro221Ala, Ile222Leu and Ile223Leu) based on CYP2C sequences were introduced in the F–G loop region by site-directed and cassette mutagenesis to improve properties for crystallization. CYP2C9 was produced in *Escherichia coli* XL1 blue cells. The cells were collected 48 h after induction and disrupted in a high salt lysis buffer by passage twice through a cell homogenizer at 10,000 pounds per square inch. CYP2C9 was purified by affinity chromatography in the presence of 0.3% detergent IGEPAL CA630 (v/v) using Ni-NTA resin (Qiagen), desalted and then directly applied to a cation exchange column in the absence of detergent.

To establish the effect of the truncation and mutagenesis, a full comparison of the activity and specificity of the CYP2C9 protein was performed. The selectivity obtained

when incubating the protein with the fluorescent probe 7-methoxy-4-trifluoromethylcoumarin against a panel of ligands was representative of the unmodified protein, and the ability to metabolize the 4' hydroxylation of diclofenac and the 6' and 7' hydroxylation of *S*-warfarin remained unchanged by the mutations introduced (data not shown).

Structure determination

Crystals of CYP2C9 were obtained by the hanging-drop vapour diffusion method, using a 1:1 ratio of protein at 40 mg ml^{−1} in a solution of 10 mM potassium phosphate, pH 7.4, 0.5 M potassium chloride, 20% (v/v) glycerol, 1 mM EDTA, 2 mM dithiothreitol against a crystallization well solution of 0.1 M Tris, pH 8.4, 15–25% (v/v) PEG 400, 5–12.5% (w/v) PEG 8000, 10% (v/v) glycerol. Crystals formed over a period of 1–4 days at 25 °C, and were frozen directly from the crystallization solution. X-ray data were collected at beam line ID14.2 at the ESRF, processed using MOSFLM²⁸, scaled and further reduced using the CCP4 suite of programs (<http://www.ccp4.ac.uk>) (Supplementary Table S1). The CYP2C9 substrate-free structure was solved by molecular replacement using the program AMORE²⁹ and the CYP2C5 structure⁷ as a search model. Model rebuilding was done using O (<http://xray.bmc.uu.se/alwyn>) and refinement was performed using CNX (<http://www.accelrys.com/products/cnx/index.html>) and REFMAC³⁰. There are two copies of CYP2C9 in the asymmetric unit, resulting in a solvent content of 70%. The resulting substrate-free structure was used in the refinement of the *S*-warfarin complex structure (Supplementary Table S1). Co-crystals of CYP2C9 and *S*-warfarin were grown using well solution supplemented with 5 mM *S*-warfarin. The *S*-warfarin complex was refined using CNX (<http://www.accelrys.com/products/cnx/index.html>) and REFMAC³⁰.

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Correspondence and requests for materials should be addressed to H.J. (h.jhoti@astex-technology.com). Coordinates have been deposited in the Protein Data Bank under accession codes 1OG2 and 1OG5.

Gene expression, amplification

PCR, thermocyclers and some more software that works with Mac OS X.

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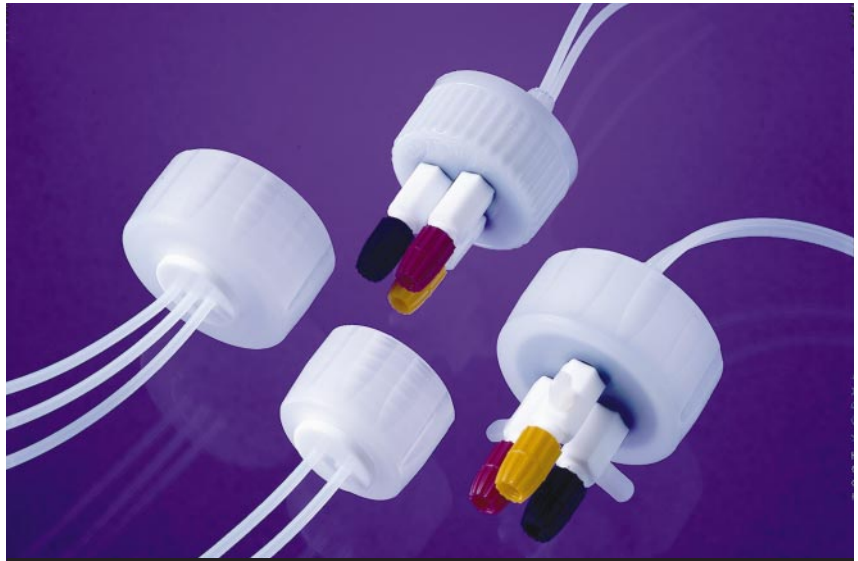
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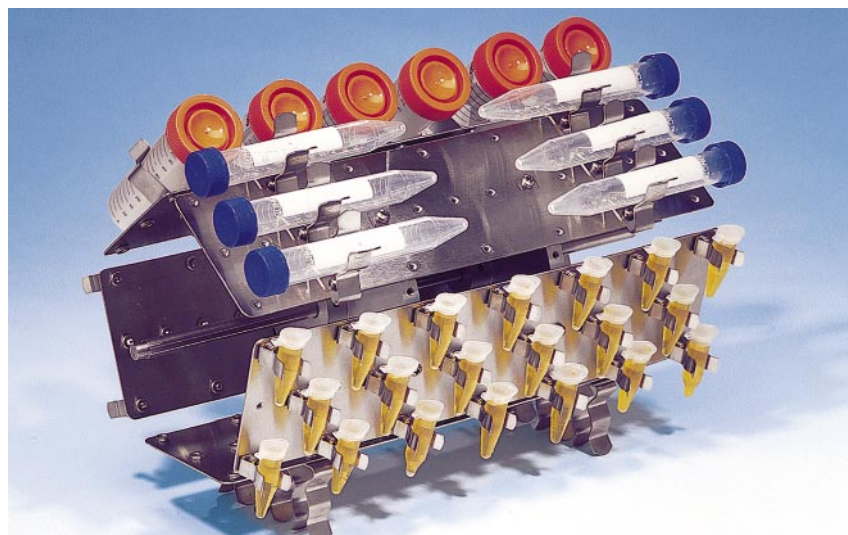
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A team effort

When Lance Armstrong won his fourth Tour de France last year, he not only followed tradition by dividing the prize money among his team-mates, he also doubled the amount to his fellow cyclists out of his own pocket. This largesse recognized that without his team's support, Armstrong wouldn't have won the race. It also demonstrates best practice in team science — rewarding the supporting cast that makes a lead author's findings possible, rather than hoarding all of the credit for oneself.

Last month the US National Institutes of Health held a symposium on how to promote selflessness in the name of team science, primarily for the benefit of biologists in the United States. But its findings (see *Nature* **424**, 1; 2003) could improve the fortunes of scientists in other disciplines or other countries who are considering joining large projects.

In some respects, this discussion is long overdue — large team efforts have dominated science in recent years. The Human Genome Project and most experiments in high-energy physics are prime examples, and such endeavours often feature a cast of hundreds. But institutions that grant scientists tenure, extend contracts and give pay rises haven't always recognized the varied contributions made to these projects.

So recognition may have to come from the scientists themselves. Although scientists may not have pockets as deep as Armstrong's, they can match his spirit in other ways. First, they can give younger scientists who make key contributions first authorship of main papers, and rotate first authorship of supporting papers among group members. They can also include details of the roles that each group member played in the work. Finally, they can remind tenure-granting institutions over and over again that a winning author is surrounded by a supporting cast, and that all should be rewarded.

Paul Smaglik
Naturejobs editor



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Transitions

◀ **Richard Kron**, professor of astronomy and astrophysics at the University of Chicago and a scientist at the Fermi National Accelerator Laboratory, became director of the Sloan Digital Sky Survey this month, succeeding **John Peoples**.

◀ **John Hood**, who is currently vice-chancellor of the University of Auckland, New Zealand, was nominated last month to become the University of Oxford's next vice-chancellor. If the university's 'parliament of dons' approves the nomination, Hood will leave New Zealand for Britain next summer, where he will take over from **Colin Lucas**.

◀ **Randal Richards** will take up the post of director of research and innovation at the UK Engineering and Physical Sciences Research Council this October, replacing **David Clark**. Richards is currently head of the department of chemistry at the University of Durham.

◀ Last month **Eugenio Coccia** became director of the National Laboratories of Gran Sasso in Italy. Coccia was most recently full professor of gravitational physics at the University of Rome Tor Vergata. He succeeds **Alessandro Bettini**.

◀ **Robert Wells**, director of the Center for Genome Research at the Institute of Biosciences and Technology, Texas A&M University, Houston, began his term as president of the Federation of American Societies for Experimental Biology this month.

◀ **David King** became director of NASA's Marshall Space Flight Center in Huntsville, Alabama, last month, after serving as deputy director. He succeeds **Art Stephenson**.

PUBLIC HEALTH

David Fleming, the new director of global health strategies for the Bill and Melinda Gates Foundation, points to the importance of electives in steering one's career.

While in his fourth year of medical school at New York University, he opted into a public-health course. He liked it so much that he went on to join a training programme at the US Centers for Disease Control and Prevention (CDC) that puts freshly minted PhDs and MDs on the 'front lines' of public health, and in 1984 became an epidemic-intelligence officer. That led to 15 years of public-health work for the state of Oregon, before he returned to the CDC in 2000 as deputy director for science and public health.

At the CDC, Fleming looked at specific diseases within the United States. At the Gates foundation, he will look more broadly, both in terms of illnesses and geography, a factor that attracted him to the job. He advises young scientists not to be too narrowly focused early in their career. "Part of your career needs to be looking at things outside your career that make you happy," he says.

CHEMICAL BIOLOGY

James Rothman will be moving uptown this year. The programme chairman of cellular biochemistry and biophysics at the Sloan-Kettering Institute in New York will establish a centre for chemical biology at Columbia University College of Physicians and Surgeons. The move from one part of Manhattan to another is fairly conventional compared with his early career, which Rothman describes as his "oddball background".

He started out with aspirations in theoretical physics, but soon learnt that there were few jobs in the field. So he opted instead for medical school, which he found wasn't right for his basic-science bent. "But it was a very helpful several years," he says, because it introduced him to biology.

Rothman found a home at last in biochemistry at Harvard University, where he could draw on his diverse education to look at basic biological processes, but in chemical terms. Stints at Stanford and Princeton taught him to look for big-picture problems and eschew short-term gratification in lieu of long-term results — something that he fears today's publish-or-perish environment is discouraging.

In the process of setting up the new centre, he hopes to help define what chemical biology is exactly; he now sees it as using chemicals to probe biological functions. He also thinks chemistry could improve understanding of gene function. Biochemistry can reproduce a process



David Fleming



James Rothman



Eric Jakobsson



Martin Nowak

outside a cell, but the regulation conferred by a cell is lost in the process — his new institute will grapple with that problem.

BIOINFORMATICS

While at university, **Eric Jakobsson** learnt that he didn't want to be boxed in. "There are enormous pressures in academia to be quite specialized," he says. "And yet so much of the future depends on working across disciplines."

Jakobsson will have the opportunity to do just that as the first director of the Center for Bioinformatics and Computational Biology at the US National Institute of General Medical Sciences (NIGMS) in Bethesda, Maryland. His background should help, as he inadvertently trained across disciplines. He first worked as an engineer for a few years before returning to academia to get a PhD in physics. But he has spent much of his career working with biologists, especially in modelling how molecules are transported within a cell.

Before moving to the NIGMS last month, Jakobsson was a professor in molecular and integrative physiology, biophysics, neuroscience and bioengineering at the University of Illinois at Urbana-Champaign, a professor at the university's Beckman Institute, and a research scientist at the National Center for Supercomputing Applications.

MATHEMATICAL BIOLOGY

Martin Nowak this month moved from one Ivy League institution to another. The Austrian-born theoretical biologist left the Institute for Advanced Study in Princeton to establish a new institute for mathematical biology at Harvard University in Cambridge, Massachusetts, funded in part by a reported \$30-million grant by Jeffrey Epstein, a New York-based investment manager.

Apart from allowing him to interact with both the department of organismic and evolutionary biology and the maths department, Nowak is looking to expand his horizons beyond traditional boundaries. One of the reasons he made the move is the prospect of more and varied local collaborations. In particular he is eager to work with **Eric Lander**, director of the Broad Institute, and Harvard cognitive scientist **Steven Pinker**.

Nowak also wants to spread the word to students that maths opens up avenues for such collaborations — and interesting careers. "One of the perspectives of the new institute is to convey the idea to mathematics students that they can have careers in many different fields," Nowak says.

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